METHODS FOR SURVEILLANCE OF ANTIMICROBIAL RESISTANT BACTERIA IN ENVIRONMENTAL WATER AND WASTEWATER

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ABSTRACT

Katy M. Brown: Methods for Surveillance of Antimicrobial Resistant Bacteria in Environmental Water and Wastewater

(Under the direction of Mark D. Sobsey)

The rise of antibiotic resistant bacteria (ARB) exacerbates the global spread and burden of infectious disease. There are no harmonized efforts for ARB surveillance and response. This study investigated the development of a culture-based indicator system for the direct detection and quantification of Extended-spectrum-β-lactamase (ESBL) and carbapenemase (KPC) producing *Enterobacteriaceae* in exposure-relevant environmental sites including hospital and domestic sewage, treated effluent, and surface waters. This was done by performing parallel assays using agar media supplemented with antibiotics, then analyzing the clinical media's performances via concentrations and proportions of ESBL-and KPC-producing target organisms found at all sample sites, and then confirming resistance profiles and identities of resulting isolates. The performance of the clinical medium was comparable to the gold standard for detection indicator organisms in all samples, excepting *E. coli* in raw sewage and surface waters. This indicator system shows promise, but more work must be done towards its iterative improvement.

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LIST OF ABBREVIATIONS

ARB	Antibiotic Resistance
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistant Gene
AST	Antimicrobial susceptibility testing
CFU	Colony Forming Units
CI	Confidence Interval
CLSI	Clinical Laboratory Standards Institute
CRE	Carbapenem Resistant Enterobacteriaceae
ECOFF	Epidemiological Cutoffs
ER	Emergency Room
ESBL	Extended Spectrum Beta Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GAL	β-D-Galactosidase
GI	Gastrointestinal
GLUC	β-D-Glucuronidase
ICU	Intensive Care Unit
KPC	Klebsiella Pneumoniae Carbapenemase
MALDI-TOF	Matrix Assisted Laser Desorption / Ionization Time of Flight
MICs	Minimum Inhibitory Concentration
MICU	Medical Intensive Care Unit
MRSA	Methicillin Resistant S*taphylococcus aureus*
MS	Mass Spectrometry
NCCLS	National Committee for Clinical Laboratory Standards
NCSU	North Carolina State University
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UNAN	United National Autonomous University of Nicaragua-León
UNC	University of North Carolina
VRE	Vancomycin-Resistant *Enterococci*
WASH	Water Sanitation and Hygiene
WHO	World Health Organization
WWTP	Waste Water Treatment Plant

CHAPTER 1: OVERVIEW

Background

Researchers have documented dramatic increases in the occurrence and types of antimicrobial resistance (AMR) worldwide. AMR is found globally, threatening the prevention and treatment of infectious diseases despite great advances in diagnostic and therapeutic interventions based on the use of antimicrobials (AMs). There are few new antimicrobial treatments in development and present use is, in many countries, excessive and indiscriminate. Without a coordinated response across the fields of medicine, agriculture, and environmental sciences, we may be on the brink of a "post-antibiotic" era[1]. Protecting the efficacy of present antibiotics is especially important, as their loss due to AMR may reverse seventy-five years of medical, agricultural, social, and economic progress made as a direct result of the advent of penicillin and subsequent antimicrobials[2]. However, strategic and effective interventions cannot be carried out until more is known about the magnitude, spread, and evolution of present AMR types, sources, and pathways.

Antimicrobials can be naturally or synthetically derived and are divided into two main categories: (1) those used in vivo for chemotherapeutic purposes, and (2) those used externally or for the decontamination of non-living material, such as sterilants, disinfectants, sanitizers, and antiseptics. Before the application of the former, abatement of infectious disease depended on the latter. An established infection could be deadly, as chemicals designed for external use were often too toxic for internal use, limiting treatment to little more than a guessing game of poultices, herbs, and amputations.

Modern antimicrobials are designed to target and hinder the intrinsic functionalities and persistence of an infectious agent without harming the host. Broadly, this can occur via inhibition or alteration of basic biological and biochemical processes that affect, and thereby deter, the development and replication of an infectious organism. Careful decisions must be made by the clinician prior to

administration of treatment, as the targets, formulation, and modality of many drugs is organism-dependent [3].

Chemotherapies for the treatment of parasitic, fungal, or viral infections are complex and difficult to develop, as eukaryotic pathogens have similar cell structure to humans and viruses utilize human cells for reproduction. Antibiotics tend to target the differences in prokaryotic cellular composition and function, such as the peptidoglycan wall found in bacteria and not in humans, as a mode of action to inhibit growth and trigger apoptosis. When the aforementioned drugs fail to eradicate the infection after being used at an established, optimal inhibitory levels, the causative microbe is considered to be resistant to treatment [4].

The global emergence and spread of AMR hinders the prevention and treatment of many important infectious diseases. First-line chemotherapies for HIV are progressively becoming ineffective due to burgeoning anti-viral resistance [1]. Increasing resistance to artemisinin-based combination antimalarial therapies may have "disastrous" impact on global malaria control and place 3.2 billion people at risk of infection, illness, and further transmission [5]. Complications from invasive antifungal resistant infections are increasing sequelae and mortality rates in patients with serious underlying diseases [6]. Multi-drug resistant tuberculosis infections have also risen, with 480,000 infections and 210,000 deaths in 2013 [1].

There are presently no globally coordinated efforts for AMR surveillance, response, and prevention. Surveillance is often relegated to academic research or accidental discovery, post-clinical analysis of specimens from critically ill patients, and healthcare based monitoring of patients and their antimicrobial resistant bacteria. This lack of coordinated surveillance globally perpetuates continued ignorance of the actual scope of AMR and hinders the development, application, and enforcement of strategic response and prevention efforts. In 2014, the World Health Organization (WHO) released a report calling for united action to combat AMR, with strong emphases on addressing the importance of antibiotic resistant bacteria (ARB)[1].

There is growing concern regarding the high levels of resistance in bacteria that cause common infections like pneumonia, gastroenteritis, uremia, and septicemia [7–9]. Alexander Fleming observed and

noted resistance in Gram-negative bacteria during initial experiments with penicillin [10]. Since then, there has been extensive documentation and research on resistance. However, little is actually known about the magnitude, global landscape, and trends of ABR due to the lack of harmonized and coordinated data on the complexity and multidimensionality of associated resistance mechanisms, selection pressures, transmission pathways, and spread.

Resistance mechanisms can co-occur and are chromosomally or plasmid mediated, depending on both the antimicrobial agent and the organism. Bacterial strains that genetically encode a particular resistance trait are said to have intrinsic resistance. Acquired resistance occurs when a mutation or horizontal gene acquisition alters the genome of selected isolates or groups of bacteria [11]. Antibiotic resistance genes (ARG) can be transferred between species, genera, and even families by different mechanisms, such as R-plasmid transfer via conjugation [12]. These common and widespread horizontal gene-transfer processes are instrumental in the emergence and spread of multi-drug resistance.

Many antibiotics work by entering the cell and inhibiting essential processes via binding to the cell wall or to key enzymes and target sites on the ribosome. An example of intrinsic resistance would be natural impermeability to certain antibiotics (e.g. Gram-negative bacteria and penicillin) or lacking the structure that the antibiotic inhibits (e.g. the absence of a typical cell wall in *mycoplasma* bacteria). If a bacterial strain is usually susceptible to an antibiotic and a mutation occurs that alters a target site, the bacteria has acquired resistance that results in the decreased affinity for the antibiotic and thus, its inability to bind and act.

Bacteria can also develop biochemical resistance pathways that overcome an antimicrobial's action, such as circumventing a growth analog by utilizing an exogenous growth factor. Efflux pumps, or transport proteins that facilitate the excretion or elimination of toxic substrates from within the cell to the environment, can make an organism resistant by simply pumping the antibiotic out. An ever-evolving resistance mechanism is the production of enzymes capable of inactivating a drug, such as hydrolysis of the β-lactam ring of an antimicrobial by β-lactamases. Thousands of these enzymes have been identified,

many conferring multi-drug resistance [3,4,13]. All of the above can co-occur, evolve and be transferred, depending on the organism and its environment.

Selection pressures, both environmental and anthropogenic, significantly influence the emergence, spread, and transfer ARB and ARG [11]. Environmental stressors, such as exposure to antibiotics and chemical pollutants, mobilize ARGs[14]. Excessive and indiscriminant use of antibiotics in medicine, agriculture (specifically, animal production and aquaponics), and households are significant anthropogenic selection pressures. Medical sectors are frequently blamed for the rise in antibiotic resistance, as health care facilities are, by nature, reservoirs of infectious disease and pharmaceutical use resulting in release[11,15,16]. However, agriculture and veterinary sectors are also to blame, as antibiotics are used extensively in food animal production and aquaculture for continued prevention of disease as well as for growth promotion [17].

Antibiotics, ARB, and ARG are released into the environment and bacterial ecosystem via human sewage (treated and untreated), animal manure, and industrial wastes [18]. A feedback loop is created that selects for and sustains ARB strains. If these strains are located in an exposure relevant reservoir, they can spread to humans and animals. Non-pathogenic bacteria can also serve a reservoir for ARG and contribute to transfer of resistance [11]. ARB infections develop, spread to others, persist, and then are treated with broader spectra of antimicrobial medication. The ARB, ARG, and antibiotic residue are then re-introduced back into the environment once excreted. These strains are then spread via several different pathways, including exposures from water, sanitation, and hygiene (WaSH) pathways, food consumption, international travel, poor domestic waste water treatment, land application of manure, and so on [13,14,17].

The large knowledge gaps about ARB hinder the development of comprehensive and strategic action plans. There are few actors with both the understanding and agency to implement such strategies[19]. Choosing and analyzing antimicrobial agents and the effects of their use in humans and animals on antimicrobial susceptibility as determined by testing are foundational to clinical diagnostic microbiology. If we are to approach this as a One Health problem, more work should be done to

integrate monitoring of exposure-relevant environmental sites for comprehensive surveillance. Extensive work has been done to standardize antimicrobial susceptibility testing methods for the selection of the most appropriate chemotherapeutic agent and to improve the clinical predictive value of the results[20,21]. However, these thresholds are for the determination of optimal clinical treatment, not optimal treatment and management of waste waters or to determine the relative magnitude of ARB in the environment, especially in exposure-relevant pathways[22]. There are a wide range of monitoring and surveillance methods for the detection and quantification of antibiotic resistance in the environment[23], but many are too capacity requiring, intensive, and complex for lower-resource areas, limiting their uptake for standardized and harmonized global surveillance[19,24].

In an effort to address this need, this Masters project focused on the evaluation of a prototype method for the direct, one-step, culture-based detection of ARB in representative waste waters and environmental samples of interest using an indicator system proposed by the World Health Organization. This was done by evaluating the direct detection and enumeration of standard chromogenic clinical agar media to determine the presence, concentration, and relative proportions of presumptive Extended Spectrum β-lactamase (ESBL) producing and presumptive carbapenemase producing (carbapenem resistant *Enterobacteriaceae*, CRE) *E. coli* and other coliforms in representative waste waters and environmental surface waters in Chapel Hill, NC. The identities and antimicrobial resistance properties of presumptive isolates of these target bacteria were further confirmed by further phenotypic and molecular analyses to determine the percentages of them correctly identified.

Project Objectives:

The goal of this research was to evaluate candidate indicators and methods for surveillance of antibiotic resistant bacteria in water and wastewaters with an emphasis on a culture-based indicator system for the enumeration of ESBL and KPC (CRE) *Escherichia coli* and other coliform bacteria. This goal was achieved by conducting field and lab-based studies designed to accomplish the following five project objectives.

Table 1.1 - Objective 1

Objective 1: Evaluate the performance of a chromogenic substrate standard clinical agar medium for use in the direct detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in representative waste waters and surface waters by comparing it to the performance of a "gold standard" chromogenic substrate agar medium for the direct detection and enumeration of *E. coli* and coliforms in environmental water, wastewater and other matrices.	
Application	Repeated, parallel assays of representative environmental and waste water samples were performed using procedures of EPA Method 1604 for membrane filtration followed by culturing on CHROMagar Orientation (clinical) agar medium and Bio-Rad Rapid'*E.coli* 2 (gold standard) agar medium. Following incubation, presumptive colonies of *E. coli* and coliforms detected by each medium were enumerated using manufactures' guides. *E. coli* and coliform concentrations were calculated as CFU/100 mL
Output	Presumptive *E. coli* and coliform concentrations from repeated, parallel assays of the same representative waste water and surface water samples, one data set for CHROMagar Orientation agar and one data set for Bio-Rad Rapid'*E. coli 2* agar.
Analysis	Use a paired Wilcoxon signed-rank test to evaluate the performance of the clinical medium (Orientation) against the detection performance of the "gold standard", environmental medium (Bio-Rad Rapid'*E. coli 2* agar) by comparing concentrations that are paired by date, sample site, and organism.

Performance Criteria	Null Hypothesis: The median difference between the two pairs of observations (CHROMagar Orientation agar concentration versus Bio-Rad Rapid'*E. coli 2* agar concentrations) is close to or equal to zero. Alternative Hypothesis: The median difference between the two paired observations is significantly greater or less than 0 with significance at α = 0.05. P< 0.05 indicates, with 95% CI, that the performance of the clinical medium is not comparable to the "gold standard" medium and not appropriate for use in the direct detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in waste water and environmental surface water matrices. P>0.05 indicates, with a 95% confidence, that the performance of the clinical medium is comparable to the "gold standard" environmental medium and capable of use in the direct detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in waste water and environmental surface water matrices.

Table 1.2 - Objective 2

Objective 2: Determine the presence, concentration, and relative proportion of presumptive Extended Spectrum β-lactamase (ESBL) producing *E. coli* and coliforms as well as presumptive carbapenemase (KPC) producing *E. coli* and coliforms (carbapenem resistant *Enterobacteriaceae* or CRE) in representative waste waters and environmental surface waters in Chapel Hill, NC, compared to the total number of *E. coli* and coliforms present in the samples analyzed.	
Application	Repeated and often parallel assays of representative waste water and environmental surface water samples were performed using the procedures of EPA Method 1604 for membrane filtration followed by plating on CHROMagar ESBL and KPC agar media. These assays were done in parallel with plating the same samples on the aforementioned Bio-Rad Rapid'*E. coli 2* agar/CHROMagar Orientation agar assays to determine percentages of totals that are resistant. Following incubation, presumptive colonies of *E. coli* and coliforms were enumerated using manufacturers' guides for colony color and appearance. Presumptive positives on the ESBL and carbapenem (KPC) media are presumed to express the resistance property of the culture medium on which they were detected. Concentrations of *E. coli* and coliform presumed to be ESBL positive and concentrations of *E. coli* and coliforms presumed to be KPC positive were calculated and expressed as in CFU/100 mL.
Output	A dataset was obtained for presumptive ESBL positive *E. coli* and coliform concentrations from repeated, parallel assays of representative waste water and environmental surface water samples. A dataset was obtained for presumptive KPC positive *E. coli* and coliform concentrations from repeated, parallel assays of representative waste water and environmental surface water samples.

Analysis	Proportions of presumptive ESBL and KPC producing bacteria relative to total *E. coli* and coliform concentrations were calculated for each resistance type, per sample, per target organism (*E. coli* and other coliforms) and per collection event relative to total *E. coli* and coliforms enumerated in the same samples. This was done by dividing the resulting concentrations of presumptive ESBL and KPC positive organisms (ESBL coliform, ESBL *E. coli,* KPC coliform, or KPC *E. coli*) by the concentrations of the same organisms (*E. coli* and coliforms) plated in the parallel on Bio-Rad Rapid'*E. coli 2* medium for total concentrations of *E. coli* and coliforms. Use a paired Wilcoxon signed-rank test to evaluate the difference in resistance profiles within each site and amongst other sites. This was performed by comparing the proportions of presumptive ESBL and KPC positive organisms within the same site. Then, average proportions of each resistance type and organism were evaluated between paired sites (e.g. Raw Sewage and Secondary Effluent proportions of ESBL or KPC positive *E. coli*). When three or more sites were compared, a Kruskal-Wallis one-way analysis of variance was performed.
Performance Criteria	Null Hypothesis: The median difference between the two pairs of observations is equal to zero. Alternative Hypothesis: The median difference between the two paired observations is greater or less than 0. ($\alpha = 0.05$) For Wilcoxon signed ranked tests, a $P < 0.05$ indicates, with 95% CI, that there is a significant difference between the medians of the two paired observations. $P > 0.05$ indicates, with a 95% CI, that there is not a significant difference between the observed proportions. With Kruskal-Wallis test, a $P > 0.05$ indicates that there is not a significant difference between the group means. A $P < 0.05$, indicates that there is a significant difference and necessitates a post-test, done using Dunn's test, to evaluate which of the observations created the significant difference in the pooled rankings.

Table 1.3 - Objective 3

Objective 3: Evaluate and attempt to validate the performance of CHROMagar ESBL agar medium for the direct detection and enumeration of Extended Spectrum β-lactam resistant *E. coli* and coliforms in representative waste water and environmental surface water sample as a component of a potential indicator system for the surveillance of ESBL production in presumptive *E. coli* and coliform isolates from samples of wastewater and environmental surface water matrices.	
Application	Repeated assays of representative environmental and waste water samples were performed using the procedures of EPA Method 1604 for membrane filtration on CHROMagar ESBL medium. Following incubation, representative colonies of *E. coli* and coliforms were selected, purified, isolated and the then frozen for future analysis.
Output	A bank of over 300 presumptive ESBL isolates of *E. coli* and coliform colonies, prepared and stored in duplicate, from representative waste water and environmental surface water samples were obtained for further analysis to determine species and phenotypic antimicrobial resistance.
Analysis	Frozen isolates of presumptive ESBL *E. coli* and coliforms were revived and subjected to VITEK 2 automated antimicrobial susceptibility testing in order evaluate and confirm reduced susceptibility to Extended Spectrum β-lactam antibiotics. MALDI-TOF MS was performed on these isolates for speciation as the basis for confirming the identities of presumptive ESBL *E. coli* and coliform isolates.
Performance Criteria	Rates of confirmed antimicrobial susceptibility profiles and speciation results were evaluated. These results provide the basis for an overall evaluation of the performance of CHROMagar ESBL agar medium for the direct culture-based detection and enumeration of ESBL *E. coli* and coliforms as fecal indicator bacteria in waste water and environmental surface water matrices

Table 1.4 - Objective 4

Objective 4: Evaluate and attempt to validate the performance of CHROMagar KPC agar medium for the direct detection and enumeration of carbapenem resistant *E. coli* and coliforms in representative waste water and environmental surface water samples as a component of a potential indicator system for the surveillance of presumptive carbapenem resistant *E. coli* and coliforms detected in wastewater and environmental surface water matrices.	
Application	Repeated and parallel assays of representative waste water and environmental water samples were performed using the procedures of EPA Method 1604 for membrane filtration on CHROMagar KPC medium. Following incubation, representative colonies of presumptive KPC *E. coli* and coliforms were selected and purified as isolates and then frozen for future analysis to determine species and antimicrobial resistance properties.
Output	A bank of over 300 presumptive *E. coli* and coliform KPC isolates, prepared and stored in duplicate, were obtained from representative wastewater and environmental water samples as purified colony isolates presumed to have carbapenem resistance traits
Analysis	Frozen presumptive KPC isolates will be revived and subjected to the Modified Hodge Test for antimicrobial susceptibility testing to phenotypically evaluate resistance profiles and thereby confirm KPC resistance. MALDI-TOF MS was performed on these isolates for speciation in order to confirm their presumptive identity.
Performance Criteria	Antimicrobial susceptibility and speciation results were to provide the basis for quantifying the relative specificity, sensitivity, positive predictive value and negative predictive value of the detection and quantification methods for KPC *E. coli* and coliforms. These results provide the basis for an overall evaluation of the performance of CHROMagar KPC agar medium for the direct culture-based detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in waste water and environmental water matrices.

Study Rationale and Design

A major gap exists in the availability of documented performance evidence for the suitability and effectiveness of simple and practical culture-based analytical methods for the direct detection and quantification of antibiotic resistant bacteria outside of clinical settings and in the environment in particular. The World Health Organization and others have proposed the development of an indicator system for the detection of ARB in water, wastewater, and other environmental media [22,25,26]. *E. coli* and coliforms are already widely accepted and used as bacterial indicators of water and wastewater to assess water- and waste-borne presence and exposures as human health risks. Therefore, the direct detection and quantification of key antimicrobial resistant members of this fecal indicator group, and specifically those having resistance due to Extended Spectrum-β-Lactamase (ESBL) activity and carbapenem resistance (called carbapenem resistant Enterobacteriaceae or CRE and also called *Klebsiella Pneumoniae* Carbapenemase (KPC)) have been suggested as candidates for such ARB analyses using standard chromogenic clinical diagnostic culture media.

To address this need, an initial collaboration was formed between members of the Sobsey Environmental Microbiology Laboratory at UNC, the School of Family Medicine at the UNC Medical School, and the Department of Microbiology at the United National Autonomous University of Nicaragua-León. The team worked together to develop, use and evaluate a simple, culture-based method for the detection and enumeration of target ARBs of concern in environmental samples. Later, the collaboration expanded to include the Department of Pathology and Laboratory Medicine at the UNC School of Medicine, the College of Veterinary Medicine at North Carolina State University, and The University of Colorado at Fort Collins.

Repeated, parallel assays involving direct, one-step membrane filtration of environmental samples onto chromogenic bacteriologic culture media described in Table 2.1. After incubation, samples were characterized via visual identification and counting of presumptive target organism colonies on agar media used during membrane filtration. The resulting assay data was analyzed and a dataset was created that included site-, event-, and organism-specific calculations and statistical analysis concerning the

presence, concentration, and relative proportion of presumptive target organisms. To better evaluate the performance of the original protocol, colonies of presumptive ESBL and KPC positive target organisms were selected, streaked to purification, and isolated. The presumptive resistance profiles and identities of these isolates were later confirmed. The result is a proof-of-concept prototype of a simple but effective monitoring method for the direct detection and enumeration of target gram-negative fecal indicator bacteria resistant to extended-β-lactams as well as reduced susceptibility to carbapenems in hotspot environmental samples of wastewater and water impacted by wastewater.

Literature Review

Present Policies and Practice of Antimicrobial Susceptibility Testing:

Antibiosis, or the antagonistic relationship between two or more organisms that is ultimately deleterious to one, is a cornerstone of the field of microbiology. Pasteur, Koch, and Ehrlich laid the groundwork for present-day antimicrobial susceptibility testing (AST) by contributing methodologies associated with the study of antibiosis[27]. Consequently, AST predates the introduction of antibiotics, including the 1874 paper by Roberts describing how liquid media containing *Penicillium glaucum* curiously inhibits contamination by other bacteria[28]. Fleming later contributed to AST by developing two broth dilution techniques, one with turbidity and the other with pH as the determinant endpoints. These methods were the forerunner to modern minimum inhibitory concentration (MIC) methodologies, including agar diffusion methods later described by Schmith and Reymann in 1940, Vincent and Vincent in 1944, Mohs in 1945, Bondi in 1947, and Frank et al in 1950[29–33]. These agar diffusion and agar dilution methods were effective but prone to error and cumbersome. Determining the MIC, or the lowest antibiotic concentration that prevents bacterial growth, was also very time-intensive. In the 1960s, critical concentration methods that separated organisms into resistant and susceptible categories were introduced, eventually being described as "breakpoint" techniques by Ericsson and Sherris in the 1970s[34].

During these nascent stages of AST, several variables that impact the replicability and performance of these techniques were documented. The WHO published a report in 1961 calling for standardization of AST methodology which subsequently led Bauer, Kirby, and others to create a globally implemented,

phenotypic AST method[35,36]. The Kirby Bauer disk-diffusion method was the basis for the formation of the National Committee for Clinical Laboratory Standards (NCCLS) in 1975. The NCCLS lead to the initiation of several other governing bodies focused on ATS standardization, including the Clinical Laboratory Standards Institute for AST in the Americas (CLSI) and the European Committee on AST (EUCAST) [27]. These organizations, and others like them, are responsible for the harmonization of MIC breakpoints across their associated geographic region. Breakpoint guidelines change in response to both the advancement of diagnostics as well as epidemiological information. The guidelines from CLSI and EUCAST, in particular, are not always in agreement, leading to discordant diagnosis and treatment, even within the same hospital and veterinary systems [37,38].

AST changed with the advent of genotypic approaches, ushering in the use and development of DNA-based assays for the detection of bacterial resistance genes[39]. Previous phenotypic methods sometimes required multiple days and other tests to truly characterize the resistance profile of the isolate. Genotypic techniques promised rapid results, but with ongoing verification and validation came a better understanding of the relationship of genetics to antimicrobial susceptibility and its limitations for use in routine diagnostics. For instance, the presence of a resistance gene many not be indicative of resistant bacteria because the gene may not be expressed. However, validation and implementation of genotypic methods lead to the discovery of a new realm of microbial ecology in the Resistome[40,41]. Present exploration of the determinants, abundance, and diversity of environmental antimicrobial resistance has led to a better understanding of non-clinical transmission and spread of AMR and also progress towards the development of much needed new antimicrobials. The incorporation of genotypic techniques into clinical diagnostic practice is still limited in many areas, potentially due to the required expertise and cost required for implementation[19].

Despite these advancements in AST, there are no standardized methodologies or coordinated efforts for the surveillance of AMR in non-clinical "hotspots," specifically the detection of ARB and ARG in exposure relevant, environmental compartments like wastewater treatment plants, pharmaceutical manufacturing effluents, and aquaculture and animal husbandry facilities[13]. There have been efforts by EUCAST to incorporate and build epidemiological cutoffs (ECOFFs), which utilize epidemiologically based

data to determine breakpoints and not therapeutic efficiency. ECOFFs separate clinically relevant bacterial populations into those with acquired resistance mechanisms (non-wild-type) from the wild-type populations that have no resistance[21]. These criteria do not include potentially emergent pathogens or ARG reservoir bacteria, limiting their full application for surveillance of known environmental hotspots. An accessible methodology is needed to give a more complete overview of the prevalence of resistance in environmental AMR hotpots, as they are critical control points for the reduction in emergence, spread, and transmission of infectious disease[1,13,19].

The Need for Standardized ARB Surveillance Systems:

Monitoring and evaluation of environmental hotspots that promote, sustain, or spread ARB and ARG are crucial for resistance control. There are no globally standardized surveillance methods for the detection and quantification of ARB in environmental samples. There are no agreed and widely implemented epidemiological or microbiological methods, protocols, and performance standards. Consequently, there is limited information and understanding of population-based ARB-associated morbidity and mortality which, in turn, hinders analysis of economic and societal impacts of ABR and interventions.

The 2014 WHO Antimicrobial Resistance Global Report on Surveillance states that ARB monitoring and evaluation are being performed in some national and regional surveillance networks but are very disparate, both in methodology and quality. In addition, only a few of the surveillance networks use the One Health approach, e.g. performing harmonized integrated surveillance of AMR in humans, food-producing animals, and food. Higher-income countries seem to have implemented AMR stewardship policies; however, they have used antibiotics extensively in multiple sectors since their advent and now require more sophisticated drugs, with broader spectrum of activity to treat even the most common infections. Lower- to middle-income countries, on the other hand, have not had wide-spread saturation for as long but are similar in the magnitude of ABR. This is a result of growing income, inadequate access to WaSH, poor waste treatment and disposal, overcrowding, and limited health-care infrastructure, which supports widespread self-prescribed use of antibiotics and spread of ARB. All of these factors then heighten the spread and evolution of resistance[14,19].

There are huge knowledge gaps and strategic evaluations of resistance trends and determinations of efficacy of resistance containment activities seem impossible[1]. High-income countries have long-established systems for routine surveillance (of which some are coordinated regionally), but all are limited geographically. Lower- to middle-income countries may have some coordinated efforts, but they are underfunded and often focus on specific diseases. To contend with the substantive divide in laboratory capacity, funding, and access to infrastructure, WHO recommended a cultured-based indicator system based on the detection of clinically significant ARB with resistance profiles appropriate for incorporation in harmonized surveillance of environmental samples[1].

Nine bacteria of international concern were identified along with reason for their urgency. *E. coli* is of concern because of its resistance to third-generation cephalosporins, including resistance conferred by ESBLs and to fluoroquinolones. *Klebsiella pneumonia* is of concern due to its resistance to third-generation cephalosporins, including resistance conferred by ESBLs, and to carbapenems. *Staphylococcus aureus* is resistant to β-lactam antibacterial drugs (methicillin, methicillin-resistant *S. aureus* [MRSA]). *Streptococcus pneumonia* is resistant or not susceptible to penicillin. Nontyphoidal *Salmonella* (NTS) and *Shigella* species are both resistant to fluoroquinolones. *Neisseria gonorrhea* was listed due to decreased susceptibility to third-generation cephalosporins[1].

Though VRE and MRSA are of high clinical importance, resistance conferred by production of ESBLs and resistance to carbapenems were chosen as priorities. Effective drugs still exist for the treatment of multi-drug resistant Gram-positive bacterial infections like VRE and MRSA. On the other hand, there has been limited pharmaceutical progress in the treatment of multi-drug resistant Gram-negative bacterial infections. β-lactams are considered to be the "workhorse" of antimicrobials due to their wide use and efficacy in clinical and veterinary settings. Carbapenems are considered to be the chemotherapeutic of last resort for the treatment of Gram-negative infections. Routine detection of ESBL producing organisms of clinical significance in human-impacted environments is relatively expected, given the known prevalence, but changes in its magnitude could elucidate overall resistance trends[12,13]. Routine detection of carbapenem resistant organisms of clinical significance in human-impacted environments, specifically drinking water sources, illustrates more dire AMR conditions requiring an urgent response[42,43].

Members of the *Enterobacteriaceae* family were suggested as indicator organisms. They are clinically and agriculturally relevant and have the following characteristics: thermotolerance, facultative anaerobic respiration, rod-shaped, Gram-negative, non-spore forming, and some that are lactose fermenting. These shared factors have defined the above as "fecal indicator" organisms and their detection is a widely used and promoted proxy to detect the presence of fecal contamination[44]. The combined coliform group includes *E. coli* and other coliforms (*Klebsiella, Enterobacter, Citrobacter, Serratia*). *E. coli* is largely fecal in origin and considered to be instrumental in community acquired infections[16,45,46]. Klebsiella (specifically *K. pneumoniae*) is associated with nosocomial infections [47–49]. Both of these organisms are priority ESBL and carbapenemase producers and their detection is potentially illustrative of overall AMR emergence, trends, and spread [1,12,50].

β-Lactam Antibiotics

In the early 1900s, Paul Ehrlich developed both the concept of selective toxicity as well as the first effective treatment for syphilis from synthesized arsphenamine. In 1928, Alexander Fleming isolated the parent compound for the antibiotic penicillin after observing a zone of inhibition on a bacteria culture plate contaminated by *Penicillium notatum.* In 1935, Gerhard Domagk developed the first sulfa-drug from a synthetically derived dye that was shown to protect mice against systemic streptococcal infections. In 1942, spurred by World War II, Ernst Chain and Howard Florey stabilized and then mass manufactured penicillin to treat bacterial infections in the British troops, subsequently releasing it to the general public, ushering in modern medical, agricultural, veterinary practice based in antibiotic therapy[3,4].

β-lactam antibiotics are the most important clinical antibiotics, as they are very highly selective and non-toxic to animal or human host cells. β-lactam antibiotics bind to transpeptidase enzyme, inhibiting the transpeptidation reaction essential to cell wall synthesis[21]. The cell wall continues to form, but is weaker due to a defective peptidoglycan backbone that would have been catalyzed by the bound, regulatory enzymes. The complex of antibiotic penicillin binding proteins (PBP) also catalyze the release of autolysins which digest the cell wall, ultimately causing the existing cell to self-degrade [3,4]

The basic formulation of penicillin is a thiazolidine ring, a β-lactam ring and an acyl (R) side chain. Over time, this natural product has become a semi-synthesized formula, undergoing several, iterative biological and chemical alterations to increase its antimicrobial spectrum, make it more stable, and to improve its pharmacokinetics [3]. In the 1950s, the original penicillin G was improved in order to contend with and treat infections caused by newly emergent penicillinase-producing staphylococci. This formula, however, was still unable to penetrate, and thus treat infections of Gram-negative rod-shaped bacteria. Ampicillin was then developed by adding an α-amino group into the benzyl chain to enhance activity against organisms such as *E. coli*. Since then, multiple, semi-synthetic β-lactam antibiotics have been developed and introduced such as cephalosporin, monobactams, and carbapenems, each designed with a broader spectrum of activity to contend with growing ABR in Gram-negative bacteria [4,51].

Extended Spectrum β-Lactamase and Carbapenem Resistant Enterobacteriaceae

Bacteria can become resistant to β-lactams via three general mechanisms: (1) blocking the interaction between the antibiotic and the target PBP, (2) altering the way in which the antibiotic binds to the PBP, and (3) inactivation of the antibiotics via hydrolysis by β-lactamases [3]. The first mechanism only occurs in Gram-negative bacteria, particularly species that have an outer membrane covering the peptidoglycan layer, such as *Pseudomonas.* Antibiotics must pass through pores in the outer membrane of Gram-negative bacteria rods and any alteration to these pores may preclude penetration of the antibiotic, resulting in its exclusion. The second mechanism is mediated by the overproduction or acquisition of new PBP as well as point mutation or recombination of existing PBP. The third mechanism occurs via the plasmid-mediated or chromosomally mediated production enzymes that hydrolyze the antibiotics, inactivating it by opening the β-lactam ring [3,4,43].

Extended-spectrum β-lactamases (ESBLs) confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and monobactam aztreonam. Since their discovery in Greece in the 1960s, close to 1000 chromosomal- and plasmid-encoded β-lactamases have been identified. ESBLs are found exclusively in Gram-negative organisms, but are very heterogeneous including TEM, TEM-1, SHV, SHV-2, CTX-M, and OXA ESBL families[43]. ESBLs do not inhibit cephamycins and carbapenems and are susceptible to β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam. Consequently, oxyimino-β-

lactam substrates, cephamycins and clavulanates are often used to test for the presence of resistance mechanisms. Identification of ESBLs can be difficult because their activity and resistance mechanisms are so heterogeneous. For instance, AmpC-type β-lactamases can provide oxyimino-β-lactam resistance, but are resistant to inhibition by clavulanate [52].

The CLSI and EUCAST recommendations have changed substantially over the years to contend with better understanding of ESBL resistance mechanisms [53]. The new MIC breakpoints for ESBL disk diffusion tests re-categorize organisms that were once defined as susceptible to intermediate or resistant[20,21]. A large number of studies have focused on the risk of transmission of ESBLs in and outside of hospital settings. In a 2009 multi-national survey of risk factors for community-acquired ESBL infections by Ben-Ami et al 2009, 35% of specimens from 938 patients were found to produce ESBL, 30% of which had no recent exposure to clinical settings or treatment[54]. A large number of studies have gone on to document the presence of ESBL-producing Enterobacteriaceae in food[55], surface water [45], and sewage [56].

More recently discovered ESBLs have been shown to confer multi-drug resistance by exhibiting hydrolytic activity against most classes of β-lactams. Treatment with carbapenem produces the most effective morbidity and mortality related outcomes from these multi-drug resistant infections [43]. Carbapenems are considered to be a last resort treatment for Gram-negative infections, as they retain activity against chromosomal cephalosporinases and ESBLs. The first carbapenem was introduced in the 1980s and has since grown to include imimpenem, meropenem, doripenem, and ertapenem. Less than thirty years later, carbapenem-resistant Enterobacteriaceae (CRE) is now a growing global threat, with estimated risks from infection at 48-71% mortality rate [57]. Carbapenemases are emerging resistance determinants for Gram-negative pathogens, mediating resistance to most carbapenems and other β-lactam antibiotics. Carbapenemase production is distinct from other mechanisms of carbapenem resistance like impaired permeability due to porin mutations. In the past decade, several different kinds of acquired carbapenemases have been identified [58].

Class A β-lactamases with carbapenemase activity encode resistance on both chromosomes and plasmids. The most clinically important Class A is the *K. pneumoniae* carbapenemase (KPC) group, as the enzymes reside on a transmissible plasmid, making it resistant to all β-lactam antibiotics. KPC-producing

Enterobacteriaceae were first reported in 2001 in North Carolina [59]. *Klebsiella* can transmit KPC to other genera of bacteria, including *Pseudomonas*, *Citrobacter*, *Salmonella*, *Serratia,* and *Enterbater* spp [58]. The susceptibility profile of KPC-producing bacteria is variable, as there are several variants of KPC, some that hydrolyze β-lactams at different rates[60].

Class D β-lactamases have a preferential ability to hydrolyze oxacillin, and are often referred to as OXA-type enzymes. OXA carbapenemases have been found in Enterobacteriaceae as well as in *Acinetobacter baumannii*. OXA carbapenemases in *A. baumanni*i are completely resistant while those in Enterobacteriaceae have variable susceptibility [58]. Class B β-lactamases (metallo-β-lactamases, MBLs) were discovered in Japan in 1991[61] and have since spread globally. The spread of this gene has been associated with nosocomial transmission and international travel[58]. Naturally occurring MBLs can be found in *Aeromonas hydrophila*, *Chryseobacterium* spp, and *Stenotrophomonas maltophilia* [13]. Acquired MBLs can be transferable between both species and genera as the genes that reside on the large plasmids and encode on integrons[58]. More recently, the New Delhi MBL (NDM-1) was discovered and may be potentially worse and more unpredictable than KPC, raising more alarm in the evolution of resistance determinants[12]. The $bla_{\text{NDM-1}}$ gene can occur in many unrelated species and is spread around the environment. It also has been shown to be frequently acquired by *K. pneumoniae* and *E. coli,* specifically in strains known to cause community-acquired infections[60]. In a 2011 study by Walsh et al, NDM-1 producing bacteria were found in tap and environmental water in New Delhi[62].

Accurately identifying CRE in clinical laboratories has proven difficult, as some KPC-producing isolates have carbapenem MICs that stay in the susceptible range [63]. In 2010, CLSI revaluated the carbapenem breakpoints for Enterobacteriaceae and recommended lowering them [20]. There has been debate regarding the suitability of CLSI standards and breakpoints for use in environmental analysis, as they are formulated around optimal chemotherapeutical levels, not transmission in environmental matrices [22]. The modified Hodge test has been used to phenotypically detect KPC activity by evaluating the reduced susceptibility of a lawn of a specific *E. coli* bacteria strain from ATCC that is mediated by the carbapenemase production of the test isolate (for more information, see Chapter 2, Methods and

Materials). A 2007 study comparing four different methods for the identification of KPC in Enterobacteriaceae by Anderson et al reported 100% sensitivity and specificity for the performance of the modified Hodge test, which was compared to PCR for the *bla*$_{KPC}$ gene[64] as the "gold standard" or benchmark. However, other studies focused on the analysis of non-clinical specimens found higher rates of false positives and false negatives[65].

AMR Hotspots

Human and animal excrement contains a diverse array of pathogens that if introduced into the environment could led to fecal-oral transmission of infectious diseases. In a best-case scenario, a governance-based system of accountability exists that manages, enforces, and monitors the safe conveyance, treatment, release, and disposal of treated sewage and manure back into the environment[66,67]. Again, best case scenario, the microbial load of the waste is reduced to a pre-standardized level of safety by a fully functioning array of treatment methods prior to its introduction back into the environment, thereby reducing the potential risk of infectious disease. This is a best case scenario, as an estimated 90% of the world's waste water is discharged untreated into rivers, lakes, and oceans[68]. The global burden of diarrheal disease is exemplary of the consequent impact of poor access to adequate water, sanitation, and hygiene[69]. Human and animal excrement can contain a broad assortment of antibiotic residues, antibiotic resistance genes, and antibiotic resistance bacterial[70]. There are no international guidelines or standards regarding their management, treatment, and disposal into the environment, exacerbating the emergence, spread and transmission of ARB and ARG globally.

Antibiotics are used excessively and indiscriminately for medical, veterinary, and agricultural purposes and result in the continual release of waste water and excrement containing ARG, ARB, and antibiotic residue into the environment. Occurrence of ARG and ARB in human and animal gut flora are not always indicative of antibiotic consumption or illness[71,72]. Consideration of the fecal-oral route of infectious disease transmission and associated critical control points for remediation of AMR hotpots are critical to addressing global AMR[18]. Critical AMR hotspots include hospitals, waste water treatment plants, waterways impacted by treated and untreated waste water release, concentrated animal food-production

operations (both land and aquaculture), and agricultural fields irrigated and fertilized with waste water[2,22,73,74]. Before considering the established hotspots, it's important to consider surface water environments in which treated and untreated wastes are discharged, as they may ultimately be the primary source of exposure, sustainment, and spread of AMR[75–77].

Bacterial aquatic environments are very diverse and dynamic and constantly evolving. Pollution from antibiotics, bactericides, and heavy metals creates selection pressure that exacerbates and sustains resistance[11]. The water, as well as the indigenous, non-pathogenic bacteria, can act as reservoirs and dissemination pathways for ARG and other genetic mobile elements. There is also a pH dependent soil / water interaction where, broadly, antibiotics can be removed from solution into a complex with the humic material sediment. Antibiotics can then be re-released into the environment if conditions change, "re-shocking" the present micro-ecology[13]. Untreated and treated wastewater is often discharged into surface water, impacting important environmental and biochemical parameters in relation to ARBs and ARGs. A 2013 study by Harnisz analyzed the changes in total and intrinsic resistance of autochthonous bacteria before and after a treated wastewater release point in Poland by performing phenotypic susceptibility tests with eight strategically selected antibiotics[78]. Her team found, like Reinthaller, that there was no clear correlation between the presence of ARB in waterways and discharge of treated wastewater. The presence of ARB in waterways is more associated with the relative quality of the waste water pre-treatment than discharge post-treatment.

The use of antimicrobial agents in medical settings influences emergence and prevalence of resistant clinical bacterial pathogens of concern. An under-evaluated aspect of these settings is the relative ARB load in hospital sewage. Assessing hospital waste water is essential, as hospital sewage is a major contributor of antibiotic resistant bacteria and antibiotic resistant genes into wastewater networks and, subsequently, to the environment[15,70,79]. Waste water treatment plants are another well-established AMR hotspot. The relative loading of antibiotics in waste-water is dependent upon the sludge-wastewater partition coefficient. Different wastewater treatment methodologies can have different microbial treatment and antibiotic removal efficiencies. Antibiotics in the right pH conditions can bind to humic

material. In a waste treatment scenario, antibiotics can bind to effluent particles, then go into dissolution into the biosolid during secondary treatment[11,16]. Land application of biosolids (including animal manure) can become a significant source of resistance transmission. The quality of both the effluent and biosolids are important in the control, release, and spread of AMR. More must be done to inform and support efforts to control and prevent the presence and release of antimicrobial agents, ARB, and ARG in human and animal wastes and environmental media and reduce animal and human exposure[18].

Proposed Indicator System

Presently there is a global paucity of antibiotic stewardship, timely AMR surveillance data, harmonized AMR detection methods, and coordinated action and governance around this growing threat to public, social, and economic health[12]. Complacency and cognitive dissonance manifests easily in the face of an enormous and potentially likely or inevitable disaster. The speed at which AMR has emerged and spread is frightening, and all actions towards its prevention and control should be done with urgency. Surveillance of ARB and ARG in the environment is daunting, but with global coordination and effort, strategic action is within our control[1].

It has been well established that raw and treated municipal and hospital sewage and aquaponics and animal production sites are AMR hotpots [15,16,70]. Evaluations of these and other impacted environmental compartments are key to determining the magnitude of AMR as well as identifying critical control points to slow its emergence and spread[18]. Methods used for routine clinical AST may not be as efficient as other methods, but they are accessible, highly standardized, and implemented globally[19–21]. A new method and indicator system should be developed based on the suggestion of the WHO and others that incorporates present strategies and targets AMR evaluation and enumeration of representative samples from important environmental hotspots. Myriad pathogens and chemotherapies to treat those pathogens exist. It is not pragmatic nor realistic to evaluate all in the surveillance of AMR in the environment. Thus, the development and evaluation of an indicator system for detection of human and animal relevant AMR pathogens is essential.

A culture-based method for the direct detection and enumeration of ESBL producers and carbapenem resistant fecal indicator organisms in environmental water, wastewater, and other exposure-relevant matrices is pertinent and needed. The use of already established fecal indicator organisms is pragmatic, as they are already institutionalized and routine in many places. ESBL production can confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and monobactam aztreonam. Oftentimes, treatment with carbapenem produces the most effective morbidity and mortality related outcomes from these multi-drug resistant infections [43]. Carbapenemase production can confer resistance to virtually all β-lactams and infections with carbapenemase-producing Enterobacteriaceae are associated with high mortality rates[58].

CHAPTER 2: MATERIALS AND METHODS

Introduction:

This methodological study focused on the development, use, and evaluation of a simple, culture-based monitoring method for the direct detection and enumeration of the target gram-negative fecal indicator bacteria having extended-β-lactam resistance as well as reduced susceptibility to carbapenems in hotspot environmental samples of wastewater and water impacted by wastewater. A summary of project elements can be found in Table 2.1, including overviews of the bacteriologic culture media that are the foci of the study, target organisms, sampling sites, and analysis strategies for the quantification and characterization of resulting assay data and bacterial isolates.

The project occurred over five phases. Figure 2.6 shows a summary timeline of each phase and the objective-relevant activities that occurred during that phase. During phase 1, repeated, parallel assays involved direct, one-step membrane filtration of environmental samples onto chromogenic bacteriologic culture described in Table 2.1. After incubation, samples were characterized via visual identification and counting of presumptive target organism colonies on agar media used during membrane filtration. Colonies of presumptive ESBL and KPC positive target organisms were selected, streaked to purification, and isolated for future confirmation of isolate identities and resistance profiles.

During phase 2, the resulting assay data was analyzed and a dataset was created that included site-, event-, and organism-specific calculations and statistical analysis concerning the presence, concentration, and relative proportion of presumptive target organisms. The third phase focused on the revival and purification of all KPC isolates, followed by confirmation of carbapenem-specific antimicrobial susceptibility and re-isolation for future characterization. Identity confirmation of presumptive of bacterial isolates detected on CHROMagar ESBL and KPC occurred in the fourth phase. Confirmation of resistance

profiles of ESBL isolates occurred in phase 5. CHROMagar ESBL and KPC media performance analysis was also finalized during the fifth phase. Project Objective summaries can be found in Tables 1.1 – 1.4.

Table 2.1 Summary of Project Elements: Culture Media, Target Organisms, Environmental Samples and Analytical Methods

Bacteriologic Culture Media	
Bio-Rad Rapid'*E. coli 2* agar	Chromogenic environmental medium validated for the detection and enumeration of *E. coli* and other coliforms bacteria in food and waste waters. This medium was used as the "gold standard" comparator to evaluate the performance of the clinical medium.
CHROMagar™ Orientation	Chromogenic clinical diagnostic medium used for the isolation and differentiation of urinary tract pathogens, both Gram positive and negative. This medium represents the baseline clinical comparator as well as the agar base for the ESBL and KPC media.
CHROMagar™ ESBL	Chromogenic medium for the detection of Gram-negative bacteria producing ESBL in stools and urine. Agar medium is made by supplementing the above Orientation media with a proprietary antibiotic supplement.
CHROMagar™ KPC	Chromogenic medium for the detection of Gram-negative bacteria with reduced susceptibility to most carbapenem agents in stools and urine. Agar medium is made by supplementing the above Orientation media with a proprietary antibiotic supplement.

Target Organisms		
E. coli	Other Non-*E. coli* coliforms (*Klebsiella, Enterobacter, Citrobacter,* and *Serratia*)	Combined Coliforms (*E. coli*+ Non-*E. coli* coliforms)

Environmental Sampling Sites			
Hospital Sewage	Community Sewage	Treated Sewage Effluent	Surface Water Impacted by Effluent

Analysis	
Quantitative Analysis (Phase 1 and 2)	Comparisons were made between Bio-Rad Rapid' *E. coli* 2 agar and CHROMagar Orientation to detect and quantify *E. coli* and coliforms in the different sample environmental matrices (Objective 1). Concentrations and proportions of presumptive ESBL and carbapenem resistant *E. coli* and coliforms in the environmental samples were made from counts taken on their respective media, CHROMagar ESBL and CHROMagar KPC (Objective 2).
Confirmation of Antimicrobial Resistance Profile (Phase 3 and 5)	For isolate originally detected on CHROMagar ESBL, reduced susceptibility to Extended-β-lactams (Cefpodoxime) and carbapenems (Imipenem) and ESBL production was evaluated via Vitek2 (Objective 3). For isolates originally detected on CHROMagar KPC, reduced susceptibility to carbapenems and KPC production was confirmed via non-automated antimicrobial susceptibility testing using the Modified Hodge test (Meropenem) for all successfully revived isolates. A select group of KPC isolates were also analyzed via the same Vitek analysis (Objective 4).
Confirmation of Isolate Identity (Phase 4)	Matrix-assisted laser desorption / ionization time of flight mass spectrometry, or MALDI-TOF MS was used for definitive species confirmation analysis of all successfully revived bacterial isolates (Objective 3 and 4).

Site Descriptions:

There were four different environmental matrices analyzed in this study: hospital sewage, municipal sewage (influent), secondary treated effluent, and surface water. Each site was located in Chapel Hill, NC and had relative hydrologic connectivity. The hospital collection sites were connected to the municipal waste water treatment plant (WWTP) where the influent and effluent samples were collected. The surface water samples were collected from a creek into which the WWTP discharges its secondary treated effluent. The creek sites were located up and downstream of the discharge point. Another surface water site is a recreational lake for which the aforementioned creek is an upstream tributary.

Hospital Sewage:

The hospital collection sites are located at the main site of UNC Hospitals on Manning Drive in Chapel Hill. This is a highly performing and ranked teaching hospital with several specialty wards. It has a multi-winged, 830-bed general medical and surgical facility. In 2015, it received around one million clinical visits, including ~38,000 admissions, 12,000 inpatients and ~17,000 outpatient surgeries, and 70,000 emergency room visits. Samples from the four collection sites were taken with the assistance of the hospital facilities staff from manholes serving four wings, each with distinct sewerage networks. The sewerage system at the first site served the medical intensive care unit (MICU) and the gastrointestinal (GI) clinic and surgical ward. The sewerage system at the second site served an intensive care unit (ICU), the burn unit, and a suite of medical laboratories. The sewerage system at the third site served another ICU, laundry facilities, and the cardiovascular clinic and surgical ward. The sewerage system at the fourth site served the emergency room, the psychiatric ward and an associated neuroscience ICU [80].

Figure 2.1 - View of Hospital Site 1

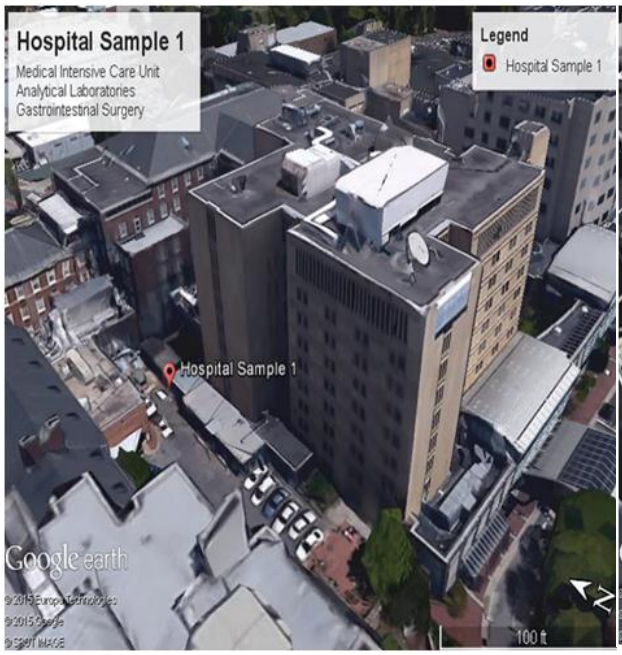


Figure 2.2 - View of Hospital Site 2

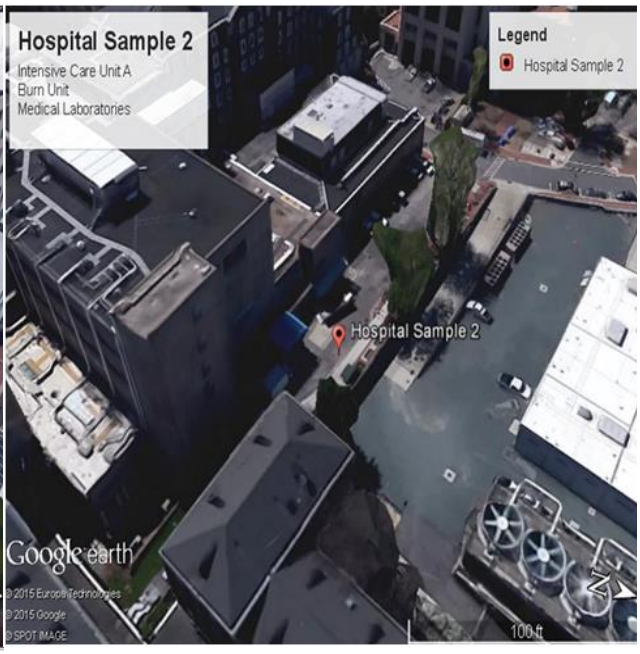


Figure 2.3 - View of Hospital Site 3

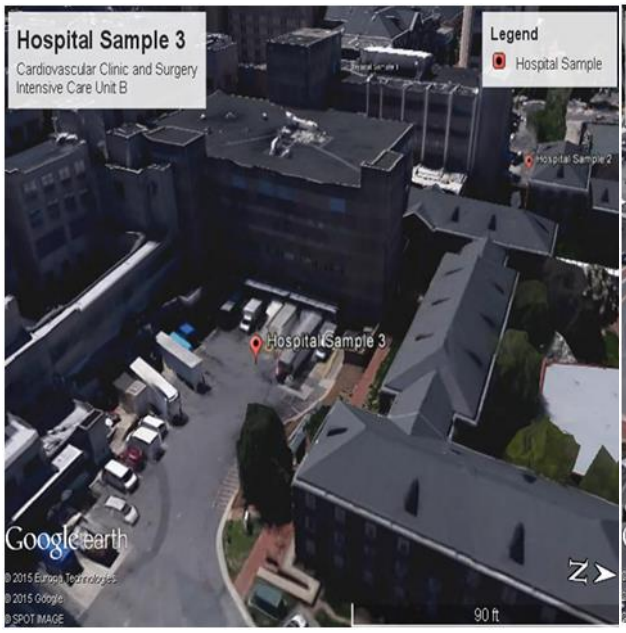
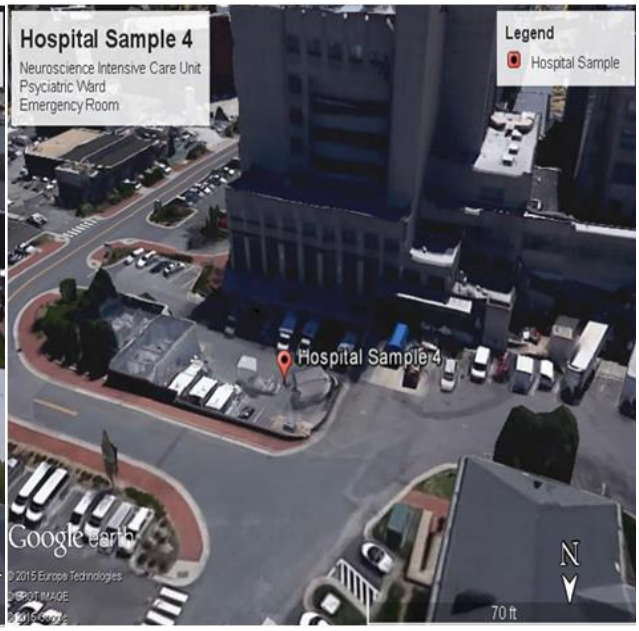


Figure 2.4 - View of Hospital Site 4



Domestic Sewage and Treated Effluent:

Samples of domestic, raw sewage and treated sewage effluent were taken at the Orange Water Sewage Authority (OWASA) at Mason Farm Wastewater Treatment Plant in Chapel Hill, NC. OWASA serves over 100,000 people in Orange County via 324 miles of wastewater collection piping. Much of this piping utilizes gravity flow, assisted by 21 pumping stations with 14 miles of pressured sewer. The peak capacity of the plant is 14.5 million gallons of wastewater per day (MGD), with the average around 8 MGD.

The treated wastewater, or secondary effluent, goes through a step-wise treatment process before release. First, during primary treatment, large grit and debris are removed from the sewage and then it is conveyed to settling tanks for primary clarification. Next, during secondary treatment, the primary effluent is pumped into a large aeration basin for aerobic biological degradation with production of settled biomass as activated sludge flocs. For secondary clarification and removal of biosolids the activated sludge is conveyed to clarifiers. The clarified effluent then undergoes rapid granular medium filtration, followed by ultraviolet light disinfection prior to release into Morgan Creek as a tributary of

Jordan Lake. Both samples were collected by OWASA staff. The raw sewage was collected after removal of large grit and debris and the disinfected secondary effluent was collected in a holding tank, prior to release.

Figure 2.5 - View of Raw Sewage, Treated Effluent, and Surface Water Site

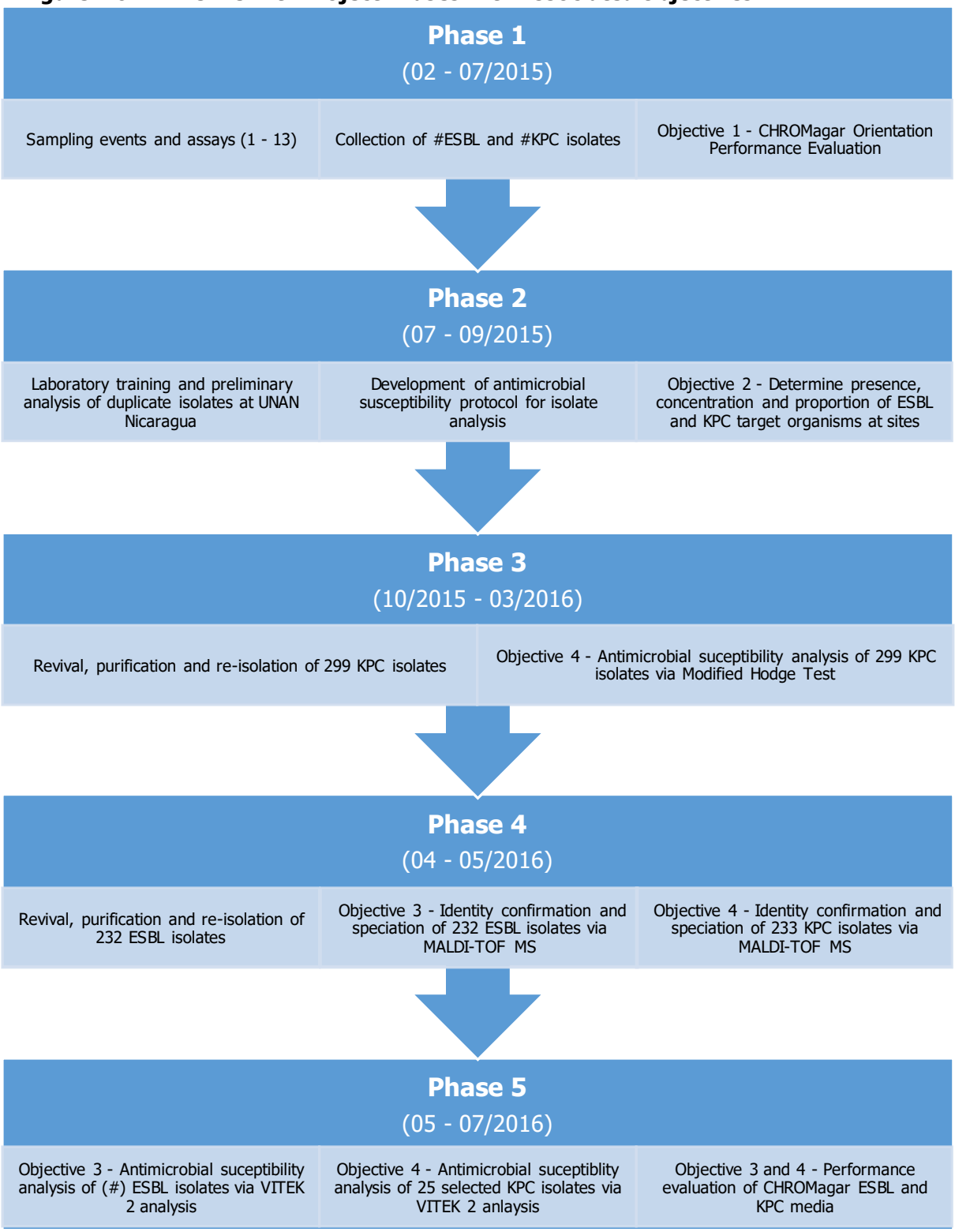
Morgan Creek and Jordan Lake:

Morgan Creek is 17 miles long and a primary tributary of Jordan Lake, a major drinking water source for the town of Cary, North Carolina. Jordan Lake was constructed as a reservoir in 1974, covers ~14000 acres, and has a history of eutrophication due to excessive nutrient levels. Collection sites for Morgan Creek were located upstream and downstream of OWASA's treated effluent release point. There were no shoreline public access points approximate to the confluence of Jordan Lake at Morgan Creek. The Jordan Lake collection site was located at a shore-line beach and boat ramp in the Jordan Lake State Recreational Area close to the intersection of Farrington Road and Martha's Chapel Road, located less than 0.5 miles southwest of the confluence.

In the flow chart below, the number of ESBL isolates on phase 5 should be specified

Project Timeline

Figure 2.6 – Timeline with Project Phases with Associated Objectives



Phase 1:

Thirteen sampling events were performed from February - July 2015 (Table 2.2). The first seven sampling events focused on the collection and analysis of samples of raw sewage and secondary treated sewage effluent taken from OWASA and from Morgan Creek, up and downstream of the effluent discharge point. Sampling events eight through ten focused on collection and analysis of samples of hospital sewage from the four, distinct sewerage networks at the UNC hospital main facility on Manning Drive. Jordan Lake sampling started in week eleven. Events eleven through thirteen focused on the concurrent collection and analysis of samples from all nine sites.

Table 2.2 – Schedule of Sampling by Site

A blue box and plus sign indicates that sampling and subsequent analysis using the original protocol. A grey box with a dash indicates that sampling and analysis did not occur.

2015 Sample Collection Dates													
Sample Site	9-Feb	16-Feb	2-Mar	17-Mar	23-Mar	31-Mar	9-Apr	6-May	11-May	18-May	26-May	1 Jun	1 – Jul
	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13
UNC Hospital Sewage Site 1	–	–	–	–	–	–	–	+	+	+	+	+	+
UNC Hospital Sewage Site 2	–	–	–	–	–	–	–	+	+	+	+	+	+
UNC Hospital Sewage Site 3	–	–	–	–	–	–	–	+	+	+	+	+	+
UNC Hospital Sewage Site 4	–	–	–	–	–	–	–	+	+	+	+	+	+
OWASA Raw Sewage	+	+	+	+	+	+	+	–	–	–	+	+	+
OWASA Secondary Effluent	+	+	+	+	+	+	+	–	–	–	+	+	+
Morgan Creek, Upstream	+	+	+	+	+	+	+	–	–	–	+	+	+
Morgan Creek, Downstream	+	+	+	+	+	+	+	–	–	–	+	+	+
Jordan Lake	–	–	–	–	–	–	–	–	–	–	+	+	+

Bacterial isolates of presumptive ESBL *E. coli* and other coliforms as well as presumptive KPC *E. coli* and other coliforms were collected during each sampling period, when available. An overview of bacterial isolates collected and specific methodological information on their selection, isolation, revival and analysis can be found in Table 2.7 and 2.8. Objective 1 (Table 1.1) was also completed during this phase, allowing for more informed analysis during Phase 2, Objective 2 (Table 1.2).

Phase 2:

The second phase of the project occurred in Fall 2015, post-laboratory training with project collaborators in the Department of Microbiology at the National Autonomous University of Nicaragua in León. Lessons learned during this training were incorporated into attempts to revive and further purify bacteria isolates collected during the first phase of work, as well as into iterative improvement in culture-based analytical methods and further evaluation of the original culture isolation and enumeration protocol.

Following the conclusion of sampling events, the resulting assay data were analyzed and datasets were created for site- event-, and organism-specific calculations and statistical analysis concerning the presence, concentration, and relative proportion of presumptive Extended Spectrum β-lactam resistant *E. coli* and other coliforms as well as presumptive carbapenem *E. coli* and other coliforms. With its completion, Objective 2 was achieved.

Phase 3:

The third phase of work focused on antimicrobial susceptibility testing of 298 successfully revived and purified KPC bacteria colony isolates via the Modified Hodge Test in order to validate the performance of CHROMagar KPC primary isolation medium to confirm and further evaluate the carbapenem resistance profiles of isolates. This analysis represents only a portion of Objective 4 and, due to funding limitations, was considered to be the last stage in the analysis associated with this project. However, new funding was obtained in March 2016 via a collaboration with Colorado State University that allowed for the implementation of Phase 4.

Phase 4:

With this new funding and with a collaboration with UNC hospitals, confirmatory identification of bacterial isolates was initiated using Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectrometry in April 2016. This required the re-revival of KPC isolates and revival and purification of previously unanalyzed ESBL isolates. Due to limited time, funding, and capacity, isolates were prioritized for analysis as representative KPC and ESBL isolates. In one month, and aided by undergraduates, a total of 233 KPC isolates were revived and 232 ESBL isolates were purified and revived and submitted for MALDI-TOF MS. Phase 4 was completed in late May 2016 and was considered to be the last phase in the analysis until more funding was identified in April via a collaboration with North Carolina State University that allowed for antimicrobial susceptibility testing of the ESBL isolates, allowing for Phase 5 and the completion of Objective 3.

Phase 5:

In late April 2016, a portion of ESBL isolates being analyzed via MALDI-TOF MS were prioritized for VITEK 2 analysis. A total of 211 isolates were submitted for analysis at NCSU. Results were returned at the end of June, allowing for merging of the data for Modified Hodge Test (MHT), VITEK 2 and MALDI-TOF MS. Data were compiled and analyzed for each culture medium to determine the rate of confirmation of presumptive identities and resistance profiles.

With this final analysis, time was taken to identify performance deficiencies and incorporate iterative improvements into a new methodology to improve performance and provide a basis for future recommendations on candidate methods for direct, culture-based environmental monitoring using a harmonized and continuous surveillance system for specified AMR traits of *E. coli* and other coliforms in the environmental samples that are the same as or consistent with the methods used in clinical and agricultural/veterinary settings and samples.

Original Protocol and Analysis Schedule:

Table 2.3 shows the original protocol and analysis schedule used to pilot the development of a culture-based indicator system for the detection and enumeration of ESBL and KPC (CRE) antimicrobial resistant enteric bacteria of health concern in fecal wastes and wastewaters. The original protocol was developed and utilized throughout the thirteen sampling periods and a description of each phase is provided throughout this chapter. The protocol has since been iteratively improved; thus it is not advised to use this exact schedule and set of procedures in practice. The key updates in the protocol will be discussed in the Recommendations section in Chapter 5.

Table 2.3 Original Method Protocol by Day

Day	Objective
Day 1	Media, Diluent and Cryopreservative Preparation: - Bio Rad Rapid' *E. coli* 2 agar medium, CHROMagar™ Orientation Medium, CHROMagar™ ESBL, CHROMagar™ KPC, Tryptic Soy Agar (Difco™), Phosphate-buffered Saline, 1X Trypticase Soy Broth, and 40% Glycerol aqueous Solution
Day 2	- Sample Collection, Transport, and Storage - Direct, One-Step Membrane Filter Method for analysis of samples on *Bio Rad Rapid' E. coli 2, CHROMagar™ Orientation Medium, CHROMagar™ ESBL, and CHROMagar™ KPC*
Day 3	- Bacteria Colony Identification and Enumeration on the Agar Media Used for Membrane Filtration Analysis - Presumptive Positive Bacteria Colony Selection and Initial Streak Plate Isolation and Purification on KPC and ESBL Agar Media
Day 4	- Second Step Colony Streak Plate Purification of Presumptive Positive Bacteria Isolates on KPC and ESBL Agar Media
Day 5	- Third Step Colony Streak Plate Purification of Presumptive Positive Bacteria on Tryptic Soy Agar (TSA)
Day 6	- Broth Culture Propagation of Selected Colony Isolates of Target Organisms from TSA Plates for All Sample Sites in Tryptic Soy Broth Medium
Day 7	- TSB Culture Isolate Supplementation with Glycerol for Frozen Storage and Later Confirmatory Identification and Antimicrobial Resistance Analysis

Media Preparation:

All culture media and stock solutions were prepared and sterilized according to manufacturer's instructions as well as by procedures in Standard Methods for The Examination of Water and Wastewater [81]. Sterilized, molten agar media were tempered to 55˚ C in a water bath prior to pouring plates. Agar medium plates were prepared in a laminar flow hood disinfected with 70% ethanol solution. Using a mechanical pipet gun and a sterile, polystyrene pipette, molten agar was dispensed into the plates at 15mL per 100mm x 15mm plate and 6 – 5mL per 60mm x 15mm plate and then allowed to solidify and dry for approximately 30 minutes. After solidifying and drying, plates were stacked upside down in sterilized baskets, and stored at 4°C for greater than 18 hours and up to 1 month before use. For more specific information on preparation of Bio-Rad Rapid'*E. coli 2*, CHROMagar™ Orientation, CHROMagar™ ESBL, CHROMagar™ KPC, Tryptic Soy Agar (Difco™) media as well as the tryptic soy broth medium and other stock solutions (phosphate buffer and aqueous glycerol solution) used in this method, see Appendix 1.

Sample Collection, Transport, and Storage

All environmental samples of wastewater and water were collected as grab samples in sterile polypropylene bottles and transported on ice in coolers to the laboratory. Grab samples from OWASA for both influent raw sewage and secondary effluent were collected by OWASA staff. Samples from Morgan Creek, both up and downstream, and Jordan Lake were collected in the field by UNC project staff. Hospital samples were obtained with the assistance of UNC hospital facilities staff. Manholes were uncovered and a weighted chain with a polypropylene sampling bottle held by a pipe clamp was lowered into the wastewater. No composite sampling was performed during the project. After collection, samples were stored at 4° C and analyzed within 48 hours. All used reusable nitrile gloves, bottles, clamps, chain and coolers were sterilized, post-collection, transport, and storage of samples for future use.

Direct, One-Step Membrane Filter Method:

Membrane filtration techniques were adapted from the procedures of EPA Method 1604 [82] and from Standard Methods [81]. The laboratory bench was disinfected with 70% ethanol solution prior to the beginning the experiment and aseptic technique was used throughout each stage of analysis.

Serial Dilutions

Samples were removed from 4°C storage and placed on ice. Samples were then diluted serially 10-fold using phosphate-buffered saline (PBS) to obtain countable colonies. See Table 2.4 for a list of typical dilutions used for each sample site and medium. Sterile bottles and pipettes were used for each dilution. Three dilutions were prepared for each sample in an effort to account for the great variability in bacteria concentrations observed at each sampling period.

The priority was to avoid confluent colony growth for purpose of obtaining isolated colonies as well as preventing the occurrence of colonies that were crowded and too numerous to count. The dilutions made were sample- and medium- specific, with more dilutions made for more highly contaminated samples as well as for plating on the agar media lacking antibiotics (e.g. for raw sewage plated on CHROMagar Orientation medium) and fewer dilutions made for samples that were expected to be less contaminated and were to be plated on media containing antibiotics (e.g. surface water samples and plating on KPC and ESBL media).

Table 2.4 - Typical Sample Dilutions Used for Membrane Filtration Analysis by Sample Site and Culture Medium

Sample Site	Bio-Rad Rapid'*E. coli 2*	CHROMagar™ Orientation	CHROMagar™ ESBL	CHROMagar™ KPC
OWASA Raw Sewage	10^{-4} 10^{-5} 10^{-6}	10^{-4} 10^{-5} 10^{-6}	10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3}
OWASA Secondary Effluent	10^{-2} 10^{-3} 10^{-4}	10^{-2} 10^{-3} 10^{-4}	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}
Morgan Creek, Upstream	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}
Morgan Creek, Downstream	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}	10^{0} 10^{-1} 10^{-2}
UNC Hospital Sewage Site 1	10^{-3} 10^{-4} 10^{-5}	10^{-3} 10^{-4} 10^{-5}	10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3}
UNC Hospital Sewage Site 2	10^{-3} 10^{-4} 10^{-5}	10^{-3} 10^{-4} 10^{-5}	10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3}
UNC Hospital Sewage Site 3	10^{-3} 10^{-4} 10^{-5}	10^{-3} 10^{-4} 10^{-5}	10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3}
UNC Hospital Sewage Site 4	10^{-3} 10^{-4} 10^{-5}	10^{-3} 10^{-4} 10^{-5}	10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3}

Membrane Filtration

The filter apparatus used for each experiment consisted of a sterile filter flask and filter funnel assembly (filter support base and filter funnel) fitted with a vacuum connection. A 0.45µm pore size, 47 mm diameter, gridded membrane filter (Millipore HA filter) was placed on the filter support base of the filter funnel assembly using sterilized forceps that were dipped in ethanol and flamed in a Bunsen burner.

After attaching the filter funnel to the filter support base containing the membrane filter, 20 milliliters of the sample was pipetted onto the filter and then vacuumed through the apparatus. The interior of the filter assembly was then rinsed with sterile phosphate buffer to wash onto the filter any bacteria that might have attached to the inner wall of the filter assembly. The membrane was aseptically removed from the filter funnel support base and transferred gridded face up to a 60 x 15 mm plate of agar medium.

Each sample was filtered in triplicate for membrane filter plating on all four agar culture media (Bio-Rad Rapid'*E. coli 2*, CHROMagar Orientation, CHROMagar ESBL, and CHROMagar KPC) at appropriate dilutions. A new, sterile filter funnel assembly was used for each water sample and a diluent negative control was run at the beginning of each membrane filtration session. Once complete, agar plates with membrane filters were then inverted and incubated at 37°C for 18 to 24 hours.

Counting and Analysis of Bacterial Colonies:

Colony Visualization and Enumeration:

After incubation, colonies on the membranes of plates were observed and counted for those having the desired color and appearance. Numbers of colonies as colony forming units (CFUs) for presumptive *E. coli* and other coliforms were totaled for each plate and recorded as discrete counts according to colony color guides provided by the manufacturer. Colony plate counts for each target organism, sample type, medium and dilution were then used to calculate target bacteria concentrations in samples as CFU/100 mL. Tables 2.5 and 2.6 were derived from manufacturers' descriptions of bacteria colony appearance.

Colony color interpretation was the primary measure of evaluation, however, other colonial characteristics such as sheen, transparency, and shape were included when needed to evaluate colonies that were similar in appearance, but did not conform exactly to manufacturers' descriptions. Other morphological characteristics were often used to differentiate between presumptive coliform and *Enterococcus* colonies on CHROMagar media.

Table 2.5 - Colony Appearance: Bio-Rad Rapid'*E. coli 2* and CHROMagar Orientation
Manufacturer's description of colony appearance assumes incubation at 37°C for 18-24 hours.

Agar Type	Microorganism	Appearance
Bio-Rad Rapid'*E. coli 2* agar	Coliforms	Blue to green
	E. coli	Violet to pink
CHROMagar ™ Orientation	Coliforms (*Klebsiella, Enterobacter, Citrobacter, Serratia*)	Metallic blue (+/- reddish halo)
	E. coli	Dark pink to reddish
	Proteus, Morganella, Providencia	Brown halo
	Proteus vulgaris	Blue with brown halo
	Pseudomonas	Translucent (+/- natural pigmentation cream to green)
	Acinetobacter	Cream
	Stenotrophomonas	Colourless
	Enterococcus	Turquoise blue
	Staphylococcu. aureus	Golden, opaque, small
	Staphylococcus epidermidis	Cream, pinpoint colonies
	Staphylococcus saprophyticus	Pink, opaque, small
	Streptococcus Group B	Light blue
	Candida albicans	Cream, pinpoint colonies

Table 2.6 - Colony Appearance: CHROMagar ESBL and CHROMagar KPC
Manufacturer's description of colony appearance assumes incubation at 37°C for 18-24 hours

Agar Type	Microorganism	Appearance
CHROMagar ™ ESBL	ESBL KEC (*Klebsiella, Enterobacter, Citrobacter)*	Metallic blue
	ESBL *E.coli*	Dark pink to reddish
	ESBL *Proteus*	Brown halo
	ESBL *Acinetobacter*	Cream
	ESBL *Pseudomonas*	Translucent (+/- natural pigmentation cream to green)
	Stenotrophomonas	Colorless
CHROMagar ™ KPC	Carbapenem^{R} KEC (*Klebsiella, Enterobacter, Citrobacter)*	Metallic blue
	Carbapenem^{R} *E. coli*	Dark pink to reddish
	Carbapenem^{R} *Pseudomonas*	Translucent (+/- natural pigmentation cream to green)
	Carbapenem^{R} *Acinetobacter*	Cream
	Stenotrophomonas	Colorless

Bacteria Concentration (CFU / 100 mL)

Bacteria concentrations for each culture medium, sample and presumptive organism identity were calculated from counts of colonies having the expected color and appearance on membranes of 60mm diameter agar medium plates. Each sample and agar medium was plated on average at 3-sample specific dilutions, each with replicates per dilution. Too numerous to count was set at 250 colonies per plate. No lower limit was established for colony counts used to determine bacteria concentration.

Calculation of colony forming unit (CFU) concentration involved adding up counts of total colonies of the expected color and appearance for all countable plates and dividing this total number of colonies by the total undiluted sample volumes they represented per counted plate (including plates with zero colonies at a sample dilution that also had some plates with visible colonies at the same sample dilution) to calculate CFU/mL, then multiplying by 100 to express the concentration as CFU/100 mL. The estimated upper and lower 95% confidence limits of these bacteria concentrations were calculated according to the

Poisson distribution from the sum of the actual colony counts for all replicates and dilutions used, taking the square root of this number and then multiplying by 2 and then adding and subtracting from the total colony count, respectively.

Calculations for Proportions of Presumptive Antimicrobial Resistant Bacteria

The proportions of presumptive ESBL and KPC positive target organisms were calculated by dividing the presumptive ESBL and KPC positive bacteria concentration of the sample obtained by plating on the ESBL and KPC media by the total concentration obtained by plating on the base medium (Bio-Rad Rapid'*E. coli 2* medium). This calculation was only performed on parallel assays, e.g. same sample, same time period, same target organisms. Bacterial concentrations from CHROMagar Orientation medium were only used if the Bio-Rad concentrations were compromised or if the medium was not available. Each occurrence is noted in Appendix 2. All hospital proportions were calculated using CHROMagar Orientation concentrations for like-organisms, as the Bio-Rad medium was not available consistently throughout weeks 8 – 10 of the project period.

Average, Standard Error, and Confidence Interval Calculations:

Averages were calculated to measure the central tendencies of presumptive concentrations and proportions of target organism. As microorganisms are assumed to be randomly distributed, Poisson standard errors were calculated for concentrations by taking the square root of (x-bar/n). Standard errors bacteria concentrations and proportions of presumptive ESBL and KPC positive organisms were calculated by dividing the associated standard deviation of the sample set by the square root of the sample number. Ninety-five-percent confidence intervals were calculated for all of the above by multiplying the standard error by 1.96 and expressed as plus or minus the average.

Normality Determination:

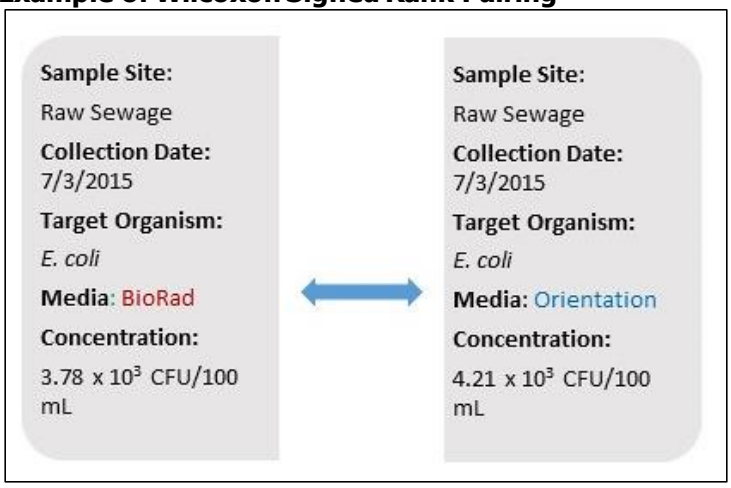
Prior to deeper analysis, the data was subjected to a Shapiro-Wilk test to evaluate its normality. This was performed in R and confirmed that non-parametric testing was required.

Paired Wilcoxon Signed Rank Tests and Mann-Whitney U-Tests:

Wilcoxon Signed Rank tests and Mann-Whitney U-tests are non-parametric tests that utilize summation of ranks and are similar to t-tests performed in normally distributed data. Wilcoxon Signed Rank tests are applied dependent or matched samples. Mann-Whitney U-tests are applied to independent samples.

Bacteria concentration and proportion data were organized by date collected, target organism, sample site, and bacteriological agar medium and maintained in Excel and then exported as a CSV file into R for analysis. Paired Wilcoxon Signed Rank tests were done to evaluate the performance of the clinical medium (Orientation) against the detection performance of the gold standard, environmental medium (Bio-Rad) in each type of representative water and wastewater sample based on concentrations as CFU / 100 mL. Site- and organism-specific paired Wilcoxon Signed Rank tests were also performed for concentrations and proportions to compare presumptive ESBL and KPC positive target organisms.

Figure 2.7 – Visual Example of Wilcoxon Signed Rank Pairing



The data for the Bio-Rad Rapid'*E. coli 2* and CHROMagar Orientation comparison paired by site and between parallel assays and target organism concentration results are shown as examples in Fig 2.7). At $\alpha = 0.05$) the null hypothesis is that the median difference between the two pairs of observations is equal to zero. Results were expressed as two-tailed P values, with values of $p < 0.05$ taken as statistically significant, for each sample site and target organism. See Objective 1 and 2 for more detail on its application.

Mann-Whitney U-Tests were performed to compare two independent sites by the same variable (e.g. compare the concentrations of presumptive KPC positive *E. coli* in Raw Sewage and Secondary Effluent). This was performed to compare proportions of presumptive ESBL and KPC positive target organisms in raw sewage and secondary effluent. Mann-Whitney U-Tests were also performed to compare target organism concentrations and presumptive ESBL and KPC positive organisms between secondary effluent and Morgan Creek upstream of the WWTP, between secondary effluent and Morgan Creek downstream of the WWTP, and between Morgan Creek up- and downstream of the WWTP. At $\alpha = 0.05$, the null hypothesis is that the median difference between the two pairs of observations is equal to zero. Results were expressed as two-tailed P values, with values of $p < 0.05$ taken as statistically significant, for each sample site and target organism.

Kruskal Wallis One-way analysis of Variance:

Bacteria concentration and proportion data were organized by date collected, target organism, sample site, and bacteriological agar medium and maintained in Excel and then exported as a CSV file into R for analysis. Kruskal Wallis was used to compare three or more independent groups. At $\alpha = 0.05$, the null hypothesis is that the mean ranks of the groups are the same. A $P>0.05$ indicates that there is not a significant difference between the group means. A $P<0.05$, indicates that there is a significant difference and necessitates a post-test, for which a Dunn's test was used to evaluate which of the observations created the difference in the pooled rankings. The post-test was performed in R.

Selection, Purification and Isolation of Colonies

Three successive purification steps by streaking onto agar media were used to isolate and purify selected bacteria colonies from membrane filters, each step with incubation at 37°C for 18 – 24 hour periods. First, well-isolated, single colonies exhibiting expected appearance of presumptive KPC and ESBL *E. coli* and other coliforms were selected from the membrane filter with a sterile wooden stick and aseptically streaked onto 100 mm x 15mm plates of KPC or ESBL medium, respectively. A maximum of 5 colonies were selected per resistance type and target organism for each sample, with one colony streaked for isolation per plate.

After incubation of the first streaked plate of each type, another purification streak was performed by selecting a single, representative colony per plate and re-streaking it for isolation on a 100mm x15 mm plate of the same medium. Next, a well isolated colony having the correct appearance from this second plate was streaked a third time, this time onto a 100 mm x15 mm tryptic soy agar plate for both ESBL and KPC isolates. After incubation on TSA, well isolated colonies were selected and inoculated into 5 mL of 1 X Tryptic Soy Broth. After overnight incubation, 1 mL aliquots of the TSB culture in 20% glycerol were prepared as alphanumerically labeled, 1.5 mL freezer tubes, organized into boxes and frozen at - 80°C for future characterization.

Overview of CHROMagar ESBL and KPC Media Performance Evaluation:

Funding for the first three phases of the project was very limited. Consequently, no confirmatory analyses were performed to assess the validity of visual colony characterizations of presumptive isolates or to further evaluate the antimicrobial susceptibility profiles of colonies prior to isolation of bacteria. Fortunately, a student research scholarship was awarded that funded travel to Nicaragua to train with project collaborators at UNAN, thereby supporting a subsequent performance evaluation of CHROMagar ESBL and KPC agar media via confirmatory analysis of duplicate isolates. During this training and analysis, problems arose that revealed methodological issues with the original protocol and assay execution. These included markedly poor rates of KPC isolate revival and discovery that several of the isolates contained mixed cultures of target organisms or were contaminated with non-target organisms.

Isolation and contamination issues, combined with the availability of funding, ultimately structured the remaining phases of the project and underlie the stepwise, and sometimes piecemeal, approach of confirmatory analysis of isolates and media performance evaluations. Though more ESBL isolates were collected in phase 1, analysis of KPC isolates was initially prioritized. Phase 3 focused heavily on the re-revival, re-purification, and antimicrobial susceptibility testing of KPC isolates due concerns that arose during phase 2. When more funding became available in March, the purified KPC isolates and a selected portion of the ESBL isolates were subjected to MALDI-TOF MS for identity confirmation in phase 4. All ESBL raw sewage and secondary effluent isolates as well as a portion of hospital sewage isolates (sites

H3 and H4) were prioritized for analysis. When other funding became available in April, phase 5 was initiated and a portion of the MALDI-TOF analyzed ESBL isolates and KPC isolates were subjected to analysis with VITEK 2.

Tables 2.7 and 2.8 provide site- and presumptive organisms-specific summaries including: the number of archived isolates originally detected on CHROMagar ESBL and KPC, the number of isolates prioritized for analysis and successfully revived, the number of isolates subjected to MALDI-TOF analysis, and the number of isolates subjected to antimicrobial resistance analysis. Resistance profiles of all ESBL isolates were confirmed via VITEK 2 automated analysis of susceptibility to Cefpodoxime, an Extended-β-lactam, and Imipenem, a carbapenem. VITEK 2 was also used to evaluate ESBL production in several of the isolates. Resistance profiles for KPC isolates were confirmed via the culture-based, non-automated Modified Hodge disk diffusion test with meropenem. A few KPC isolates were selected for further analysis via VITEK 2 and are summarized in Table 2.9. All isolate-specific results for ESBL and KPC can be found in Appendix 3 and 4, respectively. Results for VITEK 2 analysis of KPC isolates can be found in Appendix 5.

Table 2.7 – Summary of Isolates of Presumptive ESBL Positive Target Organisms and their Sources

Code	Description	Total Isolates	Total Revived	Total MALDI-TOF	Total VITEK 2
FT	HOSPITAL 1_GI+MICU_ESBL_KLEB	41	39	39	0
FE	HOSPITAL 1_GI+MICU_ESBL_ECOLI	13	8	8	0
GT	HOSPITAL 2_BURN+LAB+ICU_ESBL_KLEB	34	0	0	0
GE	HOSPITAL 2_BURN+LAB+ICU_ESBL_ECOLI	10	5	5	0
HT	HOSPITAL 3_HEART+ICU_ESBL_KLEB	26	3	3	3
HE	HOSPITAL 3_HEART+ICU_ESBL_ECOLI	14	7	7	0
IT	HOSPITAL 4_ER+NEURO_ESBL_KLEB	31	27	27	26
IE	HOSPITAL 4_ER+NEURO_ESBL_ECOLI	23	15	15	15
AT	RS DOMESTIC_ESBL_KLEB	41	41	41	41
AE	RS DOMESTIC_ESBL_ECOLI	29	20	20	17
BT	SEC EFFLUENT_ESBL_KLEB	38	35	35	33
BE	SEC EFFLUENT_ESBL_ECOLI	29	29	29	28
CT	UPSTREAM_ESBL_KLEB	28	0	0	0
CE	UPSTREAM_ESBL_ECOLI	3	3	3	0
DT	DOWNSTREAM_ESBL_KLEB	30	0	0	0
DE	DOWNSTREAM_ESBL_ECOLI	3	0	0	0
ET	JORDAN LAKE_ESBL_KLEB	0	0	0	0
EE	JORDAN LAKE_ESBL_ECOLI	0	0	0	0
All	**Total**	**393**	**232**	**232**	**163**

Table 2.8 – Summary of Isolates of Presumptive KPC Positive Target Organisms and their Sources

Code	Description	Total Isolates	Total Revived	Total MALDI-TOF	Total Modified Hodge Test
OT	HOSPITAL 1_GI+MICU_KPC_KLEB	45	45	45	45
OE	HOSPITAL 1_GI+MICU_KPC_ECOLI	11	9	0	9
PT	HOSPITAL 2_BURN+LAB+ICU_KPC_KLEB	27	27	27	27
PE	HOSPITAL 2_BURN+LAB+ICU_KPC_ECOLI	17	17	4	17
QT	HOSPITAL 3_HEART+ICU_KPC_KLEB	16	16	12	16
QE	HOSPITAL 3_HEART+ICU_KPC_ECOLI	18	11	0	11
RT	HOSPITAL 4_ER+NEURO_KPC_KLEB	26	24	12	24
RE	HOSPITAL 4_ER+NEURO_KPC_ECOLI	18	15	12	15
JT	RS DOMESTIC_KPC_KLEB	52	42	42	42
JE	RS DOMESTIC_KPC_ECOLI	35	16	12	16
KT	SEC EFFLUENT_KPC_KLEB	40	32	22	32
KE	SEC EFFLUENT_KPC_ECOLI	19	17	16	17
LT	UPSTREAM_KPC_KLEB	11	10	10	8
LE	UPSTREAM_KPC_ECOLI	0	0	0	0
MT	DOWNSTREAM_KPC_KLEB	11	11	10	11
ME	DOWNSTREAM_KPC_ECOLI	0	0	0	0
NT	LAKE_KPC_KLEB	8	8	8	8
NE	LAKE_KPC_ECOLI	0	0	0	0
All	**Total**	**354**	**300**	**232**	**298**

Table 2.9 Summary of KPC Isolates and their Sources Selected for VITEK 2 Analysis

Code	Description	Total Isolates
RT	HOSPITAL 4_ER+NEURO_KPC_KLEB	4
KT	SEC EFFLUENT_KPC_KLEB	4
LT	UPSTREAM_KPC_KLEB	4
MT	DOWNSTREAM_KPC_KLEB	9
NT	LAKE_KPC_KLEB	8
All	**Total**	**25**

The codes are based on the initial, presumptive colony color interpretation during counting, purification, and isolation (Phase 1) and not on the colony color during the secondary purification step

that occurred months later (Phases 2 – 5). Manufacturers' color guides, found in Tables 2.5 and 2.6, are the criteria used for these interpretations. The codes were maintained for consistency as well as for association with assay week during following revival, re-purification, analysis, and re-isolation. In Objective 3 and 4 Results sections in Chapter 3, VITEK identities are presented as "Number of Correctly Confirmed based on original presumptive ID prior to isolation." These results are associated with the initially assigned code and not with the colony phenotype during Phases 2 – 5.

Bacteria Isolate Revival and Re-purification

Pure cultures are necessary for the further validation of this primary colony detection and enumeration methodology via biochemical tests and other characterization. It was found necessary to re-purify all frozen isolates prior to additional characterization and validation steps in order to address mixed culture and non-target bacteria problems that occurred during the original colony isolation process. These bacteria culture purity and identity problems were due to confluent colony growth and high levels of contamination with non-target organisms. Frozen isolate aliquots were removed from the - 80°C freezer and warmed slowly in a -20 °C freezer for 2 hours.

Each isolate was then aseptically inoculated into 5 mL of 1X TSB with 10 µL plastic loops and incubated overnight at 37°C. If the broth was visibly turbid, a loopful was streaked for isolation onto 100mm x 15mm CHROMagar Orientation plates using 10 µL plastic loops. Colony morphological characteristics were documented and well isolated colonies were then re-streaked for isolation on a 100mm x 15mm KPC or ESBL plate, depending on the medium from which the isolate was originally selected. If the sample was still mixed or too concentrated to give well isolated colonies, an additional streak was performed on 100mm x 15mm MacConkey agar plates. Representative colonies considered coliforms were then streaked onto TSA, incubated, re-inoculated into 1X TSB, incubated again and re-frozen in 1 mL aliquots with 20% glycerol as in the first isolation. These re-purified isolated were saved at -80 °C for further characterization.

Identity Confirmation via MALDI-TOF MS:

Matrix-assisted laser desorption -Time of Flight Mass Spectrometry (MALDI-TOF MS) is a soft ionization process used to analyze biomolecules and large organic molecules. Successfully revived and purified bacteria isolates were subjected to MALDI-TOF MS in partnership with the clinical molecular microbiology laboratory of UNC hospital. This was done to investigate and confirm the presumptive identities of bacterial colonies originally isolated from membrane filters that were cultured on CHROMagar™ KPC and CHROMagar ESBL media.

Selected isolates were revived and purified using the aforementioned methods; isolates were streaked on TSA and incubated overnight for 18 - 24 hours at 36°C. Isolates were then re-streaked onto TSA to remove any traces of glycerol and incubated. These plates were then transported to the staff of the Clinical Molecular Microbiology Laboratory at UNC hospitals in Chapel Hill. There, they performed MALDI-TOF MS on the fresh colonies using the FDA-cleared VITEK MS per manufacture's recommendations for direct colony spotting [83]. A colony was picked from the TSA plates using a sterile toothpick and smeared on a MALDI-TOF MS plate. The inoculated plate was irradiated with a laser pulse to ionize and desorb biomolecules, such as DNA and proteins. The resulting sample ions were exposed to an electric field, accelerated into a flight tube, then separated based on relative mass and abundance of ions. The mass to charge ratio present in each sample was analyzed against a digital library of biomolecules known to be associated with different organisms.

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility testing of the selectively revived presumptive ESBL positive isolates was performed via VITEK 2 (Table 2.7). All of the antimicrobial susceptibility testing for presumptive KPC positive organisms was performed via the Modified Hodge Test, with a smaller portion selected for further VITEK 2 analysis (Tables 2.8 and 2.9).

VITEK 2 Analysis

The VITEK 2 (bioMerieux) test system was used evaluate the antimicrobial resistance profile of a selected portion of ESBL isolates and KPC isolates. This automated system evaluates susceptibility to Cefpodoxime, an Extended-β-lactam, and Imipenem, a carbapenem against associated CLSI MIC [20]. VITEK 2 was also used to evaluate ESBL production in several of the isolates.

This analysis was performed by collaborators at North Carolina State University, College of Veterinary Medicine. Isolates were transferred to their laboratory, revived by two successive streaks on to TSA. Using the FDA-cleared VITEK MS per manufacture's recommendations for direct colony spotting [83], a colony was picked from the TSA plates using a sterile toothpick and smeared on a VITEK 2 AST card. The card has a series of wells that contain substrates designed to catalyze multiple biochemical reactions, in this case the wells contained MIC of Cefpodoxime and Imipenem. When a colony is spotted on the card, the system inoculates the wells, causing a series of reactions that are then subjected to automated analysis with an optical scanner that then compares the reaction against a database of containing API reference ranges and CLSI and EUCAST appropriate MIC. The results are then reported as having resistance, intermediate resistance, and susceptibility to the antibiotics used.

ESBL production was analyzed using a test panel (card NO45) with wells containing cefepime, cefotaxime, and ceftazidime, alone and in combination with clavulanate. The wells and growth in each well is quantitatively assessed by means of an optical scanner. The ESBL production was reported as being positive or negative and assessed via analysis of the proportional reduction in growth in wells containing cephalosporin plus clavulanate compared to those containing the cephalosporin alone[84].

Modified Hodge Test for Detection of Carbapenemase Production

The Modified Hodge Test (MHT), a culture-based, antimicrobial susceptibly testing method to detect carbapenemase production of *Enterobacteriaceae*, was adapted from methodologies from the Centers for Disease Control and Clinical and Laboratory Standards Institute [20,85] Test isolates and an *E. coli* ATCC 25922 strain were revived from in 1 mL aliquots of 1 X TSB and 20% glycerol and individually inoculated on 100mm x 15mm TSA agar plates via streaking with a sterilized loop and then incubated at

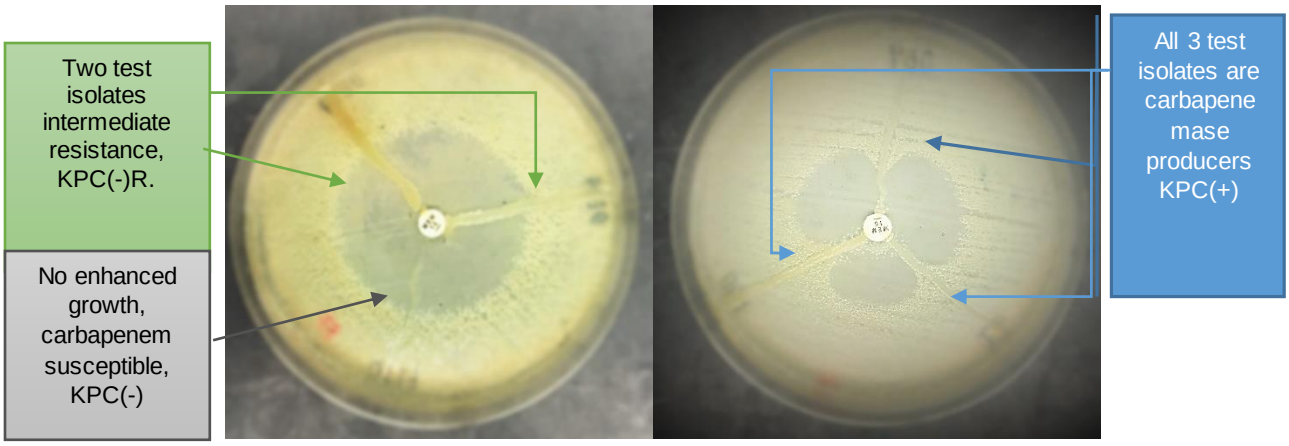
37°C for 18-24 hours. Mueller Hinton 100 x 15mm agar plates and buffered peptone water were prepared as described in Appendix 1. The next day, Mueller Hinton plates and 10 µg meropenem disks (Oxoid™) - one for each plate - were removed from the at 4°C refrigerator and allowed to equilibrate to room temperature for one hour prior to the experiment. If the agar plates had condensation, they were placed in a laminar flow hood at room temperature with lids ajar until excess surface moisture was removed. Plates were then labeled with a maximum of three test isolates per plate, spaced 120 degrees apart.

Next, a 0.5 McFarland dilution of *E. coli* ATCC 25922 was prepared by pipetting 5 mL of peptone water into a sterile 16 x 125mm glass test tube and then inoculating it with a colony from the TSA ATCC 25922 *E. coli* plate using sterile wooden stick. The tube was capped, vortexed and the turbidity was compared to a pre-prepared McFarland Standard (Remel™). Once confirmed for turbidity, the tube was briefly flamed in a Bunsen burner; a sterile cotton-tipped swab was submerged into the dilution and then swirled along the sides of the glass tube in order to remove excess liquid.

To create a lawn of the diluted *E. coli,* the cotton swab was then streaked uniformly onto the entire surface of the Mueller Hinton agar plate and then allowed to sit for 3-5 minutes. Using a sterilized needle, a 10 µg meropenem disk, was placed in the center of the prepared plate. A single, well-isolated colony was selected from the appropriate test isolate TSA plate of each sample using a 10 µL plastic loop. The picked colony was then dragged from the outer rim of the plate towards, and eventually touching, the 10 µg meropenem disk in the center of the plate. This procedure was repeated for each sample colony. A maximum of three plates were done simultaneously to ensure appropriate drying intervals. In addition, a new 0.5 McFarland dilution was prepared every 20 minutes to avoid overgrowth. The plates were inverted and incubated for 18 - 24 hours at 37°C.

After incubation, plates were analyzed for enhanced growth around the intersection of the test organism streak and the zone of inhibition in the lawn. If enhanced growth had a clover-leaf like appearance, it was indicative of positive carbapenemase production. If there was not enhanced growth, it was negative for carbapenemase production

Figure 2.8 Non-Standard Interpretation of Modified Hodge Test



The above figure shows plates that are representative of the evaluation criteria used for interpretation of carbapenemase production and carbapenem intermediate resistance and susceptibility, or KPC(+), KPC(-)R, and KPC(-), respectively. Interpretation of intermediate resistance results do not align with clinical standards and breakpoints as outlined in the CLSI [20]. These results are, however, inclusive of growing concern regarding the current limitations of said breakpoints, as their relevancy may ultimately be associated with therapeutic success and not with the evaluation of carbapenem resistance in the environmental context [1,13]. These are alternative interpretations of Modified Hodge Test to be inclusive of intermediate resistance results, as suggested in the aforementioned literature. VITEK 2 was performed on a small selection of intermediate isolates to evaluate if Imipenem resistance was also present in isolates that showed "intermediate" production of KPC on the Modified Hodge Test.

CHAPTER 3: RESULTS

Objective 1 Results:

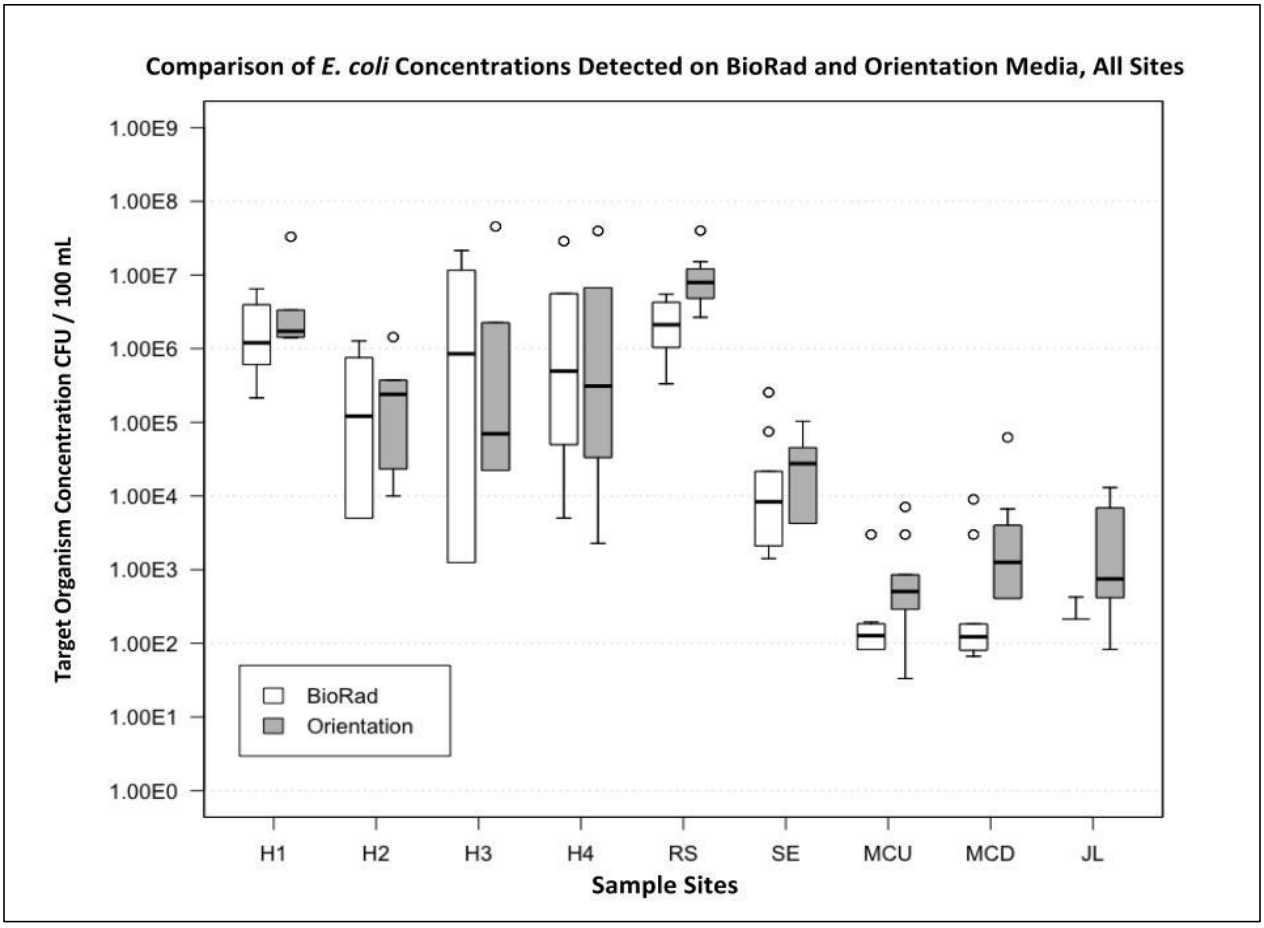
The first objective of this study was to evaluate the performance of a chromogenic substrate standard clinical agar medium for use in the direct detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in representative waste waters and surface waters by comparing it to the performance of a "gold standard" chromogenic substrate agar medium for the direct detection and enumeration of *E. coli* and coliforms in environmental water, wastewater and other matrices.

Concentrations *of E. coli,* non-*E. coli* coliforms, and combined coliforms were measured using repeated, parallel assays of environmental and wastewater samples. Each sample (Figure 3.1) was measured using CHROMagar Orientation agar, chosen as a standard clinical agar medium, and Bio-Rad Rapid'*E. coli 2* agar medium chosen as a gold standard medium. There were four sample types including hospital sewage (n=4), domestic sewage (n=1), treated sewage (n=1) and surface water (n=3) with a total of nine samples analyzed (Table 3.1).

In Figure 3.1 is shown paired *E. coli* concentration data from the clinical medium (Orientation) and the "gold standard" enviornmental medium (Bio-Rad). There is great variability in the central tendencies and ranges of *E. coli* concentration data between sample types, and expected differences in the concentrations of these bacteria according to sample type. Raw sewage samples have the highest overall median *E. coli* concentrations. The sample median concentration detected by Orientation is higher in seven out of nine sample sites when compared to the sample median concentration detected by Bio-Rad. When viewed as a subset of sample types, each of the hospital sewage sites has an outlier concentration on Orientation, but there is greater overall dispersion of concentations on Bio-Rad as indicated by the larger sizes of the interquartile ranges on this medium compared to the Orientation in

some of the samples analyzed. In the Morgan Creek surface water sites, there is a noticable difference in concentrations between the two media, with higher concentrations on Orientation. The Bio-Rad concentrations for Jordan Lake lack an actual box due to the low sample number (N=3) and concentrations were higher on Orientation.

Figure 3.1 - Box plot comparison of *E. coli* concentrations from parallel assays on CHROMagar Orientation and Bio-Rad Rapid' *E. coli 2* agar media



In Table 3.1 is shown Wilcoxon Signed Rank tests data to evaluate the performance of the clinical medium (Orientation) against the detection performance of the gold standard environmental medium (Bio-Rad) in each type of representative water and wastewater sample based on presumptive *E. coli* concentrations as CFU/100 mL. Data were paired by site and by sampling time from parallel assays. The performance of the clinical medium was comparable to the gold standard environmental medium and capable of effective use in the direct detection and enumeration of *E. coli* as fecal indicator bacteria in

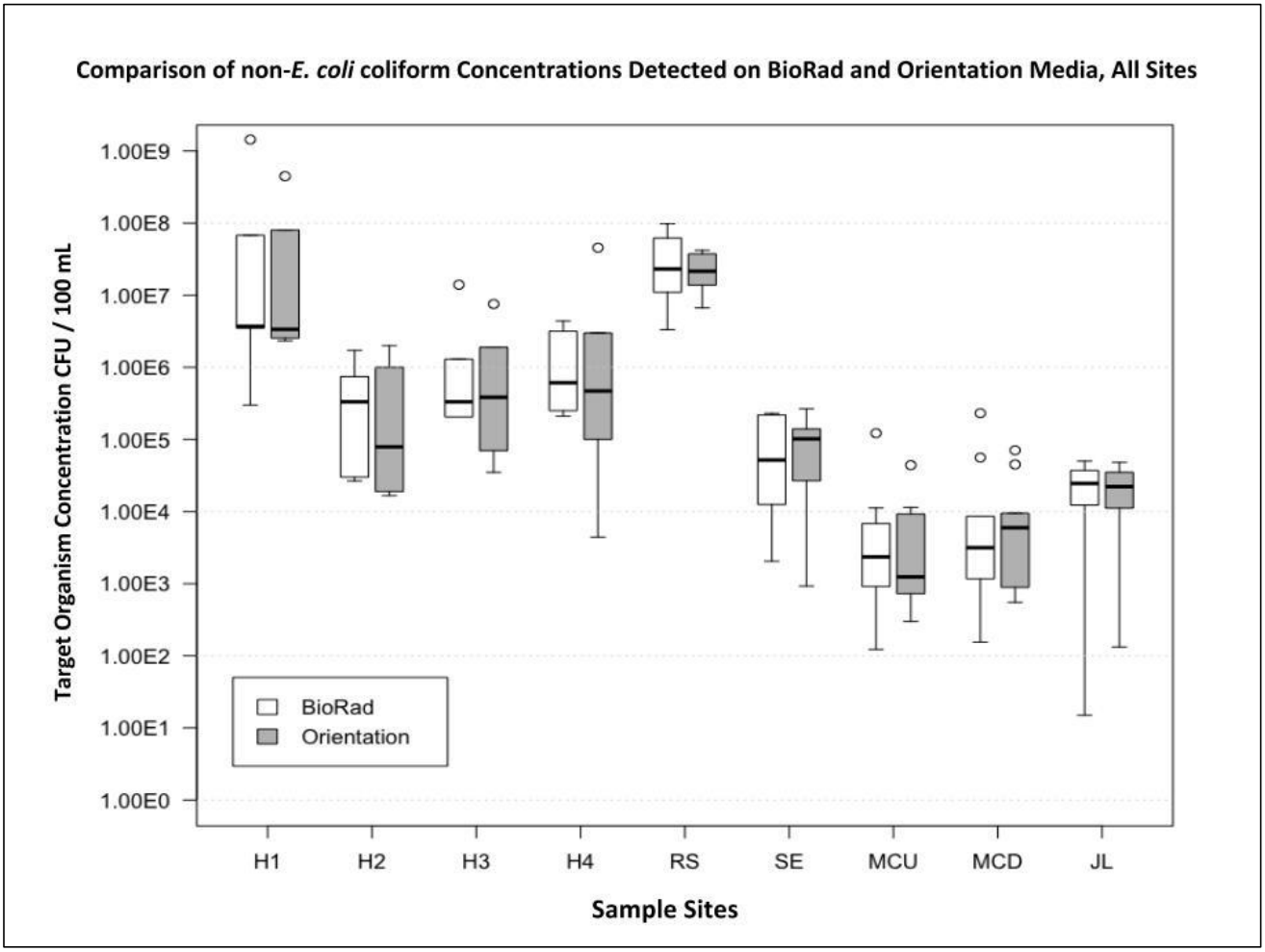
hospital sewage, treated sewage effluent and one of the three surface water matrices at the 95% confidence level. However, the performance of the clinical medium was not comparable to the gold standard environmental medium by giving higher *E. coli* concentrations in some samples.

Table 3.1 Bio-Rad Rapid'*E. coli 2* v. CHROMagar Orientation Performance Comparison for the Enumeration of *E. coli* by Wilcoxon Signed Rank Test (α = 0.05)

Sample Type	Sample Site	N	P value
Hospital Sewage	H1 GI + MICU	4	0.181
	H2 Burn + Lab + ICU	4	0.125
	H3 Heart + ICU	4	0.181
	H4 ER + Neuro	5	0.313
Domestic Sewage	Raw Sewage	8	0.031 *
Treated Sewage	Secondary Effluent	10	0.160
Surface Water	Morgan Creek Upstream	10	0.015 *
	Morgan Creek Downstream	10	0.006 *
	Jordan Lake	3	0.250

In Figure 3.2 are shown paired non-*E. coli* coliform (such as *Klebsiella, Enterobacter, Citrobacter, Serratia)* concentration data from the clinical medium (Orientation) and the gold standard environmental medium (Bio-Rad). There is less variability in the dispersion of the coliform concentration data between sample types, when compared to the *E. coli* data of Figure 3.2. Raw sewage still has the highest overall median concentration, but Hospital Site 1 has very right, upper skew and more variable concentration data, with high outliers that exceed raw sewage concentrations in other samples analyzed. However, the sample median coliform concentrations detected by Orientation are numerically and visually similar to those on Bio-Rad agar, with the exception of Hospital Site 2 in which the median concentration on Orientation is higher than on Bio-Rad. In the Morgan Creek surface water sites, there are high outliers in each site that overlap or exceed concentrations in secondary effluent. Coliform concentrations in Jordan Lake also show agreement, despite the low sample number (N=3).

Figure 3.2 Box plot comparison of other coliform concentrations from parallel assays on CHROMagar Orientation and Bio-Rad Rapid' *E. coli 2* agar media



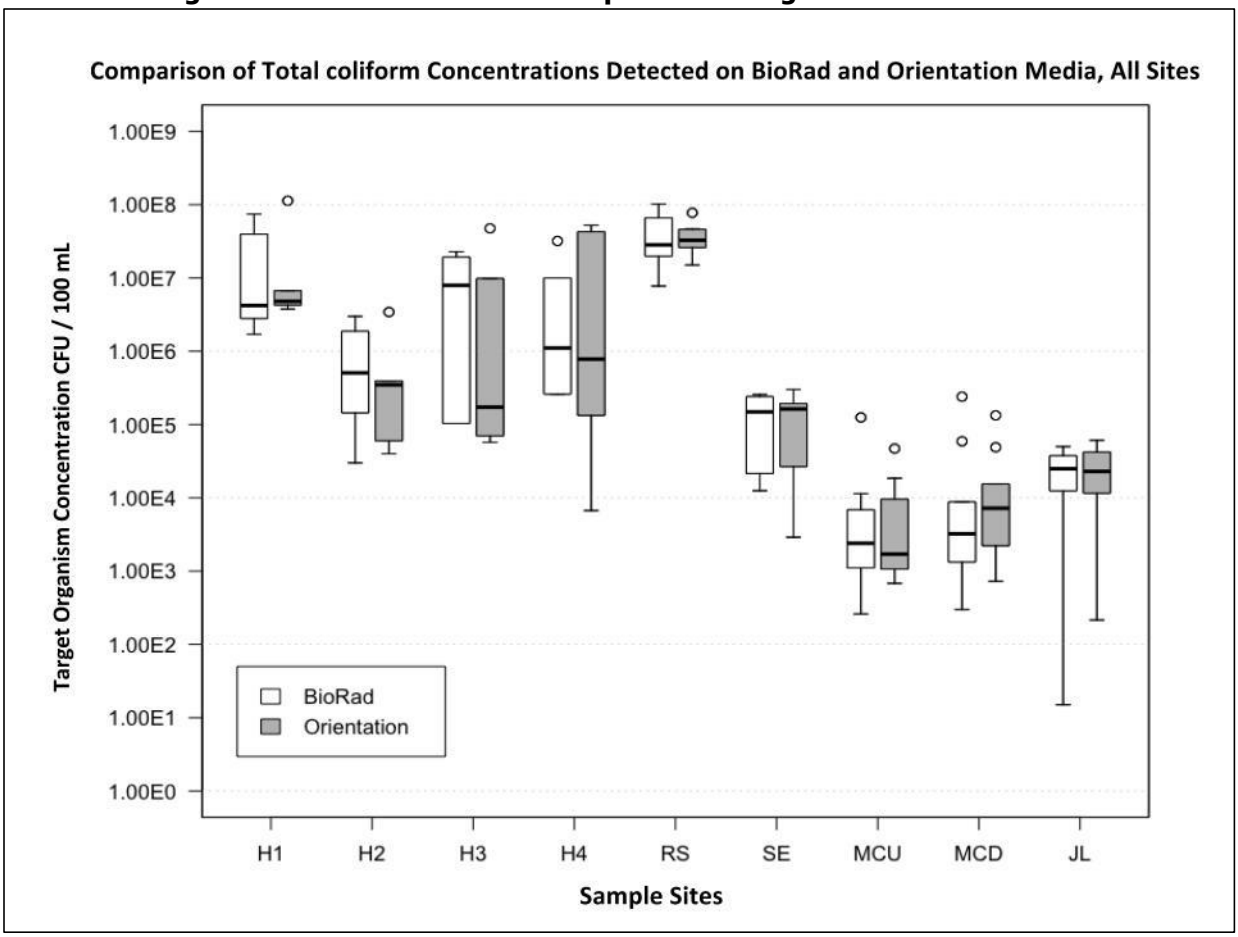
In Table 3.2 are the Wilcoxon Signed Rank tests data that evaluate the performance of the clinical medium (Orientation) against the detection performance of the gold standard, environmental medium (Bio-Rad) in each type of representative water and wastewater sample based on presumptive non-*E. coli* coliform concentrations as CFU/100 mL. Data were paired by site and by sample date for parallel assays. The performance of the clinical medium was comparable to the gold standard environmental medium. Based on these results of non-significant differences in other coliform concentrations compared to Bio-Rad agar, Orientation agar is capable of use in the direct detection and quantification of other coliforms as fecal indicator bacteria in hospital sewage, raw sewage, secondary effluent, and surface water at the 95% confidence level.

Table 3.2 Bio-Rad Rapid'*E. coli 2* v. CHROMagar Orientation Performance Comparison for the detection of other coliforms by Wilcoxon Signed Rank Test (α = 0.05)

Sample Type	Sample Site	N	P value
Hospital Sewage	H1 GI + MICU	5	1.000
	H2 Burn + Lab + ICU	5	0.625
	H3 Heart + ICU	5	1.000
	H4 ER + Neuro	5	1.000
Domestic Sewage	Raw Sewage	10	0.625
Treated Sewage	Secondary Effluent	10	0.846
Surface Water	Morgan Creek Upstream	10	0.770
	Morgan Creek Downstream	10	0.432
	Jordan Lake	3	0.500

In Figure 3.3 are shown paired Combined coliform concentrations (non-*E. coli* coliform concentrations added to *E. coli* concentrations from the clinical medium (Orientation) and the gold standard environmental medium (Bio-Rad). Raw sewage still has the highest overall median Combined coliform concentration, with the exception of one Hospital Site 1 sample as an outlier value. The sample median concentrations of Combined coliforms detected by Orientation and Bio-Rad are similar, with the exception of the Hospital Site 3 where it is higher on Bio-Rad. In the Morgan Creek surface water sites Combined coliform concentrations are similar on Orientation and Bio-Rad, and for both media there are outliers at each site that overlap or exceed concentrations in secondary effluent. The Jordan Lake Combined concentrations are in agreement for both media, despite the low sample number (N=3).

Figure 3.3 Box plot comparison of Combined coliform concentrations from parallel assays on CHROMagar Orientation and Bio-Rad Rapid' *E. coli 2* agar media



In Table 3.3 are shown Wilcoxon Signed Rank test results from comparing the performance of the clinical medium (Orientation) against the performance of the gold standard, environmental medium (Bio-Rad) based on Combined coliform concentrations as CFU/100 mL in each type of representative water and wastewater sample. Data were paired by site and by sample date in parallel assays. The performance of the clinical medium was comparable to the gold standard environmental medium and capable of effective use in the direct detection and quantification of Combined coliforms as fecal indicator bacteria in hospital sewage, raw sewage, secondary effluent, and surface water at 95% confidence level

Table 3.3 Bio-Rad Rapid'*E. coli 2* v. CHROMagar Orientation Performance Comparison for the detection of Combined coliforms by Wilcoxon Signed Rank Test (α = 0.05)

Sample Type	Sample Site	N	P value
Hospital Sewage	H1 GI + MICU	4	1.000
	H2 Burn + Lab + ICU	4	0.313
	H3 Heart + ICU	4	0.625
	H4 ER + Neuro	5	0.438
Domestic Sewage	Raw Sewage	8	0.578
Treated Sewage	Secondary Effluent	10	1.000
Surface Water	Morgan Creek Upstream	10	0.846
	Morgan Creek Downstream	10	0.846
	Jordan Lake	3	0.750

Objective 2 Results

Objective 2 was completed during Phase 2 and focused on determining the presence, concentration, and relative proportion of presumptive Extended Spectrum β-lactam resistant *E. coli* and coliforms as well as presumptive carbapenem resistant *E. coli* and coliforms in representative samples of hospital sewage, municipal sewage influent and secondary treated effluent as well as surface waters up and downstream from the effluent discharge point.

The following analysis was done to evaluate resistance profiles within each site and amongst other sites. Wilcoxon-signed rank tests were performed to compare pared observations within the same site. Mann-Whitney U tests were used to compare observations between sites. When three or more sites were compared, a Kruskal-Wallis one-way analysis of variance was performed.

Hospital Sewage

As shown in Table 3.4, the average concentrations of presumptive ESBL and KPC positive *E. coli* in H1 were 1.26×10^6 and 4.73×10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 7.00% and 2.50%, respectively. The average concentrations of presumptive ESBL and KPC positive *E. coli* in H2 were 2.93×10^4 and 8.30×10^3 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 3.22% and 1.48%, respectively. The average concentrations of presumptive ESBL and KPC positive *E. coli* in H3 were 3.57×10^4 and 1.56×10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 24.19% and 13.82%, respectively. The average concentrations of presumptive ESBL and KPC positive *E. coli* in H4 were 5.65×10^5 and 6.81×10^4 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 61.61% and 2.15%, respectively. The average concentrations of presumptive ESBL and KPC positive *E. coli* in all hospital sewage samples combined were 5.00×10^5 and 1.80×10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 27.0% and 4.8%, respectively.

Table 3.4 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive *E. coli* in Hospital Sewage Samples

Hospital 1 (GI and MICU)		Bio-Rad Rapid Agar	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
E. coli N = 5	Average	2.27E+06	1.26E+06	4.73E+05	7.00%	2.50%
	Standard Error	7.54E+02	5.02E+02	3.08E+02	3.10%	1.10%
	95% Confidence ±	1.48E+03	9.83E+02	6.03E+02	6.00%	2.20%
Hospital 2 (Burn, Lab, ICU)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
E. coli N = 5	Average	3.79E+05	2.93E+04	8.30E+03	3.22%	1.48%
	Standard Error	3.08E+02	7.65E+01	4.07E+01	1.69%	0.72%
	95% Confidence ±	6.04E+02	1.50E+02	7.99E+01	3.32%	1.41%
Hospital 3 (ICU, cardiac, laundry)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
E. coli N = 5	Average	7.73E+06	3.57E+04	1.56E+05	24.19%	13.82%
	Standard Error	1.61E+03	8.45E+01	1.76E+02	13.52%	6.75%
	95% Confidence ±	3.15E+03	1.66E+02	3.46E+02	26.51%	13.24%
Hospital 4 (ER, Neuro)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
E. coli N = 6	Average	6.98E+06	5.65E+05	6.81E+04	61.61%	2.15%
	Standard Error	1.18E+03	3.07E+02	1.07E+02	34.57%	0.75%
	95% Confidence ±	2.32E+03	6.02E+02	2.09E+02	67.76%	1.47%
All Hospitals Combined		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
E. coli N = 20	Average	4.30E+06	5.00E+05	1.80E+05	27.0%	4.8%
	Standard Error	5.18E+02	1.58E+02	9.48E+01	11.6%	2.0%
	95% Confidence ±	1.02E+03	3.10E+02	1.86E+02	22.7%	3.9%

*** The proportions of ESBL and KPC E. coli were calculated using CHROMagar Orientation in the denominator, as there was one week without a parallel assay on Bio-Rad Rapid'E. coli 2 agar. Consequently, the average concentration, standard error, and confidence intervals for Bio-Rad combined analysis were calculated with an N = 16.*

In H1 (N=5), H2 (N=5), and H3 (N=5) the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive *E. coli* showed that the distribution of the two groups did not differ

significantly at a 95% CL (Table 3.5). The same concentration analysis for H4 showed that the *E. coli* concentrations did differ significantly (N = 6, P = 0.031), at 95% CL.

In Table 3.5 is also shown the Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive *E. coli* in the hospital sites. Tests for H1 (N=5), H2 (N=5), and H3 (N=5) showed that the proportions of the *E. coli* did not differ significantly at 95% CL. In H4, the proportions of the *E. coli* did differ significantly (N = 6, P = 0.031), at the 95% CL.

Table 3.5 - Comparison of Presumptive ESBL and KPC Positive *E. coli* in Hospital Sewage: Site-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

	***E. coli* Concentration**			**% *E. coli***		
Sample	**N**	**V**	**P-value**	**N**	**V**	**P-value**
H1	5	12	0.313	5	12	0.313
H2	5	10	0.1	5	10	0.100
H3	5	9	0.813	5	7	0.584
H4	6	21	**0.031**	6	21	**0.031**

As shown in Table 3.6, the average concentrations of presumptive ESBL and KPC positive Non-*E. coli* coliforms in H1 were 5.38 x 10^6 and 3.00 x 10^6 CFU / 100 mL, respectively. The average proportions of ESBL and KPC non-*E. coli* coliforms were 36.2% and 26.30%, respectively. The average concentrations of presumptive ESBL and KPC positive non-*E. coli* coliforms in H2 were 1.62 x 10^5 and 3.95 x 10^4 CFU / 100 mL, respectively. The average proportions of ESBL and KPC non-*E. coli* coliforms were 21.74% and 27.59%, respectively. The average concentrations of presumptive ESBL and KPC positive non-*E. coli* coliforms in H3 were 1.36 x 10^5 and 3.18 x 10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC non-*E. coli* coliforms were 49.35% and 46.55%, respectively. The average concentrations of presumptive ESBL and KPC positive non-*E. coli* coliforms in H4 were 8.07 x 10^5 and 2.02 x 10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC non-*E. coli* coliforms were 70.12% and 13.11%, respectively. The average concentrations of presumptive ESBL and KPC positive non-*E. coli* coliforms in all hospital sewage samples combined were 1.62 x 10^6 and 8.89 x 10^5 CFU / 100 mL,

respectively. The average proportions of ESBL and KPC non-*E. coli* coliforms were 44.4% and 28.4%, respectively.

Table 3.6 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Non-*E. coli* coliforms in Hospital Sewage Samples

Hospital 1 (GI and MICU)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Other Coliforms N = 6	Average	3.04E+08	5.38E+06	3.00E+06	36.20%	26.30%
	Standard Error	7.80E+03	9.47E+02	7.07E+02	13.40%	15.50%
	95% Confidence ±	1.53E+04	1.86E+03	1.38E+03	26.20%	30.40%
Hospital 2 (Burn, Lab, ICU)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Other Coliforms N = 6	Average	5.70E+05	1.62E+05	3.95E+04	21.74%	27.59%
	Standard Error	3.38E+02	1.64E+02	8.11E+01	10.46%	16.75%
	95% Confidence ±	6.62E+02	3.22E+02	1.59E+02	20.50%	32.83%
Hospital 3 (ICU, cardiac, laundry)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Other Coliforms N = 6	Average	3.16E+06	1.36E+05	3.18E+05	49.35%	46.55%
	Standard Error	7.95E+02	1.51E+02	2.30E+02	26.00%	29.25%
	95% Confidence ±	1.56E+03	2.95E+02	4.52E+02	50.96%	57.33%
Hospital 4 (ER, Neuro)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Other Coliforms N = 6	Average	1.73E+06	8.07E+05	2.02E+05	70.12%	13.11%
	Standard Error	5.88E+02	3.67E+02	1.84E+02	22.65%	5.02%
	95% Confidence ±	1.15E+03	7.19E+02	3.60E+02	44.39%	9.84%
All Hospitals Combined		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Other coliforms N = 24	Average	7.74E+07	1.62E+06	8.89E+05	44.4%	28.4%
	Standard Error	1.97E+03	2.60E+02	1.92E+02	9.7%	9.1%
	95% Confidence ±	3.86E+03	5.09E+02	3.77E+02	19.0%	17.8%

*** The proportions of ESBL and KPC Non-E. coli coliform were calculated using CHROMagar Orientation in the denominator, as there was one week without a parallel assay on Bio-Rad Rapid'E. coli 2 agar. Consequently, the average concentration, standard error, and confidence intervals for Bio-Rad combined analysis were calculated with an N = 20.*

In H1 (N=6), H2 (N=6), and H3 (N=6), the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive non-*E. coli* coliforms showed that the distribution of the two groups did not differ significantly at a 95% CL (Table 3.7). The same concentration analysis for H4 showed that the non-*E. coli* coliform concentrations did differ significantly (N = 6, P = 0.031), at the 95% CL.

Table 3.7 also shows Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive non-*E. coli* coliforms in the hospital sites. Tests for H1 (N=6), H2 (N=6), and H3 (N=6) showed that the proportions of the non-*E. coli* coliforms did not differ significantly at a 95% CL. In H4, the proportions of the non-*E. coli* coliforms did differ significantly (N = 6, P = 0.031), at the 95% CL.

Table 3.7 - Comparison of Presumptive ESBL and KPC Positive Non-*E. coli* Coliforms in Hospital Sewage: Site-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

	Non-*E. coli* coliforms Concentration			**% Non-*E. coli* coliforms**		
Sample	**N**	**V**	**P-value**	**N**	**V**	**P-value**
H1	6	18	0.156	6	16	0.313
H2	6	15	0.438	6	13	0.688
H3	6	5	0.59	6	7	1
H4	6	21	**0.031**	6	21	**0.031**

As shown in Table 3.8, the average concentrations of presumptive ESBL and KPC positive combined coliforms in H1 were 4.40×10^6 and 1.87×10^6 CFU / 100 mL, respectively. The average proportions of ESBL and KPC combined coliforms were 26.9% and 18.50%, respectively. The average concentrations of presumptive ESBL and KPC positive combined coliforms in H2 were 1.57×10^5 and 5.57×10^4 CFU / 100 mL, respectively. The average proportions of ESBL and KPC combined coliforms were 9.25% and 22.06%, respectively. The average concentrations of presumptive ESBL and KPC positive combined coliforms in H3 were 1.32×10^5 and 4.71×10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC combined coliforms were 39.82% and 31.89%, respectively. The average concentrations of presumptive ESBL and KPC positive combined coliforms in H4 were 1.37×10^6 and 2.71

x 10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC combined coliforms were 52.28% and 5.93%, respectively. The average concentrations of presumptive ESBL and KPC positive combined coliforms in all hospital sewage samples combined were 1.51 x 10^6 and 6.47 x 10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC combined coliforms were 33.0% and 18.9%, respectively.

Table 3.8 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Combined Coliforms in Hospital Sewage

Hospital 1 (GI and MICU)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Combined coliforms N = 5	Average	2.11E+07	4.40E+06	1.87E+06	26.90%	18.50%
	Standard Error	2.30E+03	9.38E+02	6.11E+02	6.50%	10.20%
	95% Confidence ±	4.50E+03	1.84E+03	1.20E+03	12.80%	20.00%
Hospital 2 (Burn, Lab, ICU)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Combined coliforms N = 5	Average	1.01E+06	1.57E+05	5.57E+04	9.25%	22.06%
	Standard Error	5.02E+02	1.77E+02	1.06E+02	5.12%	15.68%
	95% Confidence ±	9.84E+02	3.48E+02	2.07E+02	10.03%	30.74%
Hospital 3 (ICU, cardiac, laundry)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Combined coliforms N = 5	Average	9.67E+06	1.32E+05	4.71E+05	39.82%	31.89%
	Standard Error	1.55E+03	1.63E+02	3.07E+02	23.52%	23.02%
	95% Confidence ±	3.05E+03	3.19E+02	6.02E+02	46.10%	45.12%
Hospital 4 (ER, Neuro)		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Combined coliforms N = 6	Average	8.71E+06	1.37E+06	2.71E+05	52.28%	5.93%
	Standard Error	1.32E+03	4.78E+02	2.12E+02	17.36%	2.34%
	95% Confidence ±	2.59E+03	9.37E+02	4.16E+02	34.02%	4.59%
All Hospitals Combined		**Bio-Rad Rapid Agar**	**CHROMagar ESBL**	**CHROMagar KPC**	**% ESBL**	**% KPC**
Combined coliforms N = 21	Average	1.00E+07	1.51E+06	6.47E+05	33.0%	18.9%
	Standard Error	7.69E+02	2.68E+02	1.76E+02	8.0%	6.8%
	95% Confidence ±	1.51E+03	5.25E+02	3.44E+02	15.7%	13.4%

*** The proportions of ESBL and KPC Non-E. coli coliform were calculated using CHROMagar Orientation in the denominator, as there was one week without a parallel assay on Bio-Rad Rapid'E. coli 2 agar. Consequently, the average concentration, standard error, and confidence intervals for Bio-Rad combined analysis were calculated with an N = 17.*

In H1 (N=5), H2 (N=5), and H3 (N=5) the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive combined coliforms showed that the distribution of the two groups did not differ significantly at a 95% CL (Table 3.9). The same concentration analysis for H4 showed that the combined coliform concentrations did differ significantly (N = 6, P = 0.031), at a 95% CL.

Table 3.5 also shows Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive combined coliforms in the hospital sites. Tests for H1 (N=5), H2 (N=5), and H3 (N=5) showed that the proportions of the combined coliforms did not differ significantly at a 95% CL. In H4, the proportions of the combined coliforms did differ significantly (N = 6, P = 0.031), at a 95% CL.

Table 3.9 - Comparison of Presumptive ESBL and KPC Positive Combined Coliforms in Hospital Sewage: Site-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

	Combined Coliforms Concentration			% Combined Coliforms		
Sample	**N**	**V**	**P-value**	**N**	**V**	**P-value**
H1	5	12	0.313	5	11	0.438
H2	5	10	0.625	5	8	1
H3	5	7	1	5	8	1
H4	6	21	**0.031**	6	21	**0.031**

Raw Sewage

As shown in Table 3.10, the average concentrations of presumptive ESBL and KPC positive *E. coli* in municipal sewage were 2.70 x 10^5 and 6.43 x 10^4 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 12.19% and 2.76%, respectively. The average concentrations of ESBL and KPC positive other coliforms were 1.17 x 10^6 and 2.37 x 10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive other coliforms were 4.40% and 1.02%, respectively. The average concentrations of ESBL and KPC positive combined coliforms were 1.44 x 10^6 and 3.01 x 10^5 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive combined coliforms were 3.09% and 0.74%, respectively.

Table 3.10 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Target Organisms in Municipal Sewage

Raw Sewage		Bio-Rad Rapid Agar	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
E. coli N = 10	Average	2.58E+06	2.70E+05	6.43E+04	12.19%	2.76%
	Standard Error	5.68E+02	1.64E+02	8.02E+01	7.10%	1.30%
	95% Confidence ±	1.11E+03	3.22E+02	1.57E+02	13.90%	2.50%
Other Coliforms N = 10	Average	3.34E+07	1.17E+06	2.37E+05	4.40%	1.02%
	Standard Error	1.83E+03	3.42E+02	1.54E+02	1.3%	0.3%
	95% Confidence ±	3.58E+03	6.71E+02	3.02E+02	2.6%	0.6%
Combined coliforms N = 10	Average	4.22E+07	1.44E+06	3.01E+05	3.09%	0.74%
	Standard Error	2.30E+03	3.80E+02	1.73E+02	0.7%	0.1%
	95% Confidence ±	4.50E+03	7.44E+02	3.40E+02	1.3%	0.3%

In Table 3.11, the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive *E. coli* in municipal sewage showed that the distribution of the two groups did differ significantly (N = 10, P = 0.010), at a 95% CL (Table 3.11). The same concentration analysis for the other coliforms and combined coliforms in municipal sewage showed that the non-*E. coli* coliforms did

differ significantly (N = 10, P = 0.002) and the combined coliform concentrations did differ significantly (N = 10, P = 0.002), at a 95% CL.

The Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive target organisms in municipal sewage showed that the proportions of the *E. coli* did differ significantly (N = 10, P = 0.006), the proportions of non-*E. coli* coliforms did differ significantly (N = 10, P = 0.006), and the proportions of combined coliforms did differ significantly (N = 10, P = 0.002), at a 95% CL (Table 3.11).

Table 3.11 - Comparison of Presumptive ESBL and KPC Positive Target Organisms in Municipal Sewage: Organism-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

***E. coli* Concentration**			**Non-*E. coli* coliforms Concentration**			**Combined Coliforms Concentration**		
N	V	P-value	N	V	P-value	N	V	P-value
10	52	**0.010**	10	55	**0.002**	10	55	**0.002**
% *E. coli*			**% Non-*E. coli* coliforms**			**% Combined Coliforms**		
N	V	P-value	N	V	P-value	N	V	P-value
10	53	**0.006**	10	55	**0.006**	10	55	**0.002**

Secondary Effluent

As shown in Table 3.12, the average concentrations of presumptive ESBL and KPC positive *E. coli* in secondary effluent were 2.89 x 10^2 and 1.53 x 10^2 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 3.50% and 1.78%, respectively. The average concentrations of ESBL and KPC positive other coliforms were 1.11 x 10^3 and 1.10 x 10^3 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive other coliforms were 1.60% and 1.55%, respectively. The average concentrations of ESBL and KPC positive combined coliforms were 1.39 x 10^3 and 1.26 x 10^3 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive combined coliforms were 1.31% and 1.23%, respectively.

Table 3.12 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Target Organisms in Secondary Effluent

Secondary Effluent		Bio-Rad Rapid Agar	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
E. coli N = 10	Average	3.93E+04	2.89E+02	1.53E+02	3.50%	1.78%
	Standard Error	6.27E+01	5.38E+00	3.92E+00	1.3%	0.9%
	95% Confidence ±	1.23E+02	1.05E+01	7.68E+00	2.6%	1.7%
Other Coliforms N = 10	Average	9.58E+04	1.11E+03	1.10E+03	1.60%	1.55%
	Standard Error	9.79E+01	1.05E+01	1.05E+01	0.4%	0.4%
	95% Confidence ±	1.92E+02	2.06E+01	2.06E+01	0.7%	0.7%
Combined coliforms N = 10	Average	1.35E+05	1.39E+03	1.26E+03	1.31%	1.23%
	Standard Error	1.16E+02	1.18E+01	1.12E+01	0.3%	0.3%
	95% Confidence ±	2.28E+02	2.31E+01	2.20E+01	0.6%	0.5%

In Table 3.13, the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive *E. coli* in secondary effluent showed that the distribution of the two groups did differ significantly (N = 10, P = 0.049), at a 95% CL (Table 3.13). The same concentration analysis for the

other coliforms and combined coliforms in municipal sewage showed that the non-*E. coli* coliforms did not differ significantly (N = 10, P = 0.846) and the combined coliform concentrations did not differ significantly (N = 10, P = 0.625), all at a 95% CL.

The Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive target organisms in secondary effluent showed that the proportions of the *E. coli* did not differ significantly (N = 10, P = 0.064), the proportions of non-*E. coli* coliforms did not differ significantly (N = 10, P = 0.846), and the proportions of combined coliforms did not differ significantly (N = 10, P = 0.770), all at a 95% CL (Table 3.13).

Table 3.13 - Comparison of Presumptive ESBL and KPC Positive Target Organisms in Secondary Effluent: Organism-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

***E. coli* Concentration**			**Non-*E. coli* coliforms Concentration**			**Combined Coliforms Concentration**		
N	V	P-value	N	V	P-value	N	V	P-value
10	47	**0.049**	10	30	0.846	10	33	0.625
% *E. coli*			**% Non-*E. coli* coliforms**			**% Combined Coliforms**		
N	V	P-value	N	V	P-value	N	V	P-value
10	46	0.064	10	30	0.846	10	31	0.770

Morgan Creek, Upstream of WWTP

As shown in Table 3.14, the average concentrations of presumptive ESBL and KPC positive *E. coli* in Morgan Creek, upstream of WWTP were 2.48 x 10^1 and 9.13 x 10^1 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 3.18% and 3.19%, respectively. The average concentrations of ESBL and KPC positive other coliforms were 1.77 x 10^2 and 3.89 x 10^2 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive other coliforms were 2.11% and 1.43%, respectively. The average concentrations of ESBL and KPC positive combined coliforms were 2.02x 10^2 and 4.81 x 10^2 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive combined coliforms were 2.01% and 1.56%, respectively.

Table 3.14 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Target Organisms in Morgan Creek, Upstream of WWTP

Morgan Creek Upstream		Bio-Rad Rapid Agar	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
E. coli N = 10	Average	4.02E+02	2.48E+01	9.13E+01	3.18%	3.19%
	Standard Error	6.34E+00	1.58E+00	3.02E+00	1.60%	3.00%
	95% Confidence ±	1.24E+01	3.09E+00	5.92E+00	3.20%	5.80%
Other Coliforms N = 10	Average	1.52E+04	1.77E+02	3.89E+02	2.11%	1.43%
	Standard Error	3.89E+01	4.21E+00	6.24E+00	1.10%	0.60%
	95% Confidence ±	7.63E+01	8.24E+00	1.22E+01	2.20%	1.20%
Combined coliforms N = 10	Average	1.56E+04	2.02E+02	4.81E+02	2.01%	1.56%
	Standard Error	3.95E+01	4.49E+00	6.93E+00	1.00%	0.70%
	95% Confidence ±	7.73E+01	8.80E+00	1.36E+01	2.00%	1.30%

In Table 3.15, the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive *E. coli* in Morgan Creek, upstream of WWTP showed that the distribution of the two groups did not differ significantly (N = 10, P = 1.000), at a 95% CL (Table 3.15). The same concentration analysis for the other coliforms and combined coliforms in municipal sewage showed that the non-*E. coli* coliforms did not differ significantly (N = 10, P = 0.813) and the combined coliform concentrations did not differ significantly (N = 10, P = 0.813), all at a 95% CL.

The Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive target organisms in secondary effluent showed that the proportions of the *E. coli* did not differ significantly (N = 10, P = 1.000), the proportions of non-*E. coli* coliforms did not differ significantly (N = 10, P = 0.636), and the proportions of combined coliforms did not differ significantly (N = 10, P = 0.722), all at a 95% CL (Table 3.15).

Table 3.15 - Comparison of Presumptive ESBL and KPC Positive Target Organisms in Morgan Creek, Upstream: Organism-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

***E. coli* Concentration**			**Non-*E. coli* coliforms Concentration**			**Combined Coliforms Concentration**		
N	V	P-value	N	V	P-value	N	V	P-value
10	7.5	1.000	10	20	0.813	10	20	0.813
% *E. coli*			**% Non-*E. coli* coliforms**			**% Combined Coliforms**		
N	V	P-value	N	V	P-value	N	V	P-value
10	8	1.000	10	27	0.636	10	26	0.722

Morgan Creek, Downstream of WWTP

As shown in Table 3.16, the average concentrations of presumptive ESBL and KPC positive *E. coli* in Morgan Creek, downstream of WWTP were 6.03 x 10^1 and 3.17 CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 11.55% and 1.89%, respectively. The average concentrations of ESBL and KPC positive other coliforms were 3.70 x 10^2 and 1.42 x 10^2 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive other coliforms were 1.92% and 0.87%, respectively. The average concentrations of ESBL and KPC positive combined coliforms were 4.30x 10^2 and 1.45 x 10^2 CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive combined coliforms were 2.18% and 0.87%, respectively.

Table 3.16 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Target Organisms in Morgan Creek, Downstream of WWTP

Morgan Creek Downstream		Bio-Rad Rapid Agar	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
E. coli N = 10	Average	1.29E+03	6.03E+01	3.17E+00	11.55%	1.89%
	Standard Error	1.14E+01	2.46E+00	5.63E-01	6.40%	1.60%
	95% Confidence ±	2.23E+01	4.81E+00	1.10E+00	12.50%	3.20%
Other Coliforms N = 10	Average	3.12E+04	3.70E+02	1.42E+02	1.92%	0.87%
	Standard Error	5.59E+01	6.08E+00	3.77E+00	0.63%	0.41%
	95% Confidence ±	1.10E+02	1.19E+01	7.38E+00	1.23%	0.81%
Combined coliforms N = 10	Average	3.25E+04	4.30E+02	1.45E+02	2.18%	0.87%
	Standard Error	5.70E+01	6.56E+00	3.81E+00	0.71%	0.43%
	95% Confidence ±	1.12E+02	1.29E+01	7.46E+00	1.38%	0.85%

In Table 3.17, the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive *E. coli* in Morgan Creek, downstream of WWTP showed that the distribution of the two groups did not differ significantly (N = 10, P = 0.107), at a 95% CL (Table 3.17). The same concentration analysis for the other coliforms and combined coliforms in municipal sewage showed that the non-*E. coli* coliforms did not differ significantly (N = 10, P = 0.477) and the combined coliform concentrations did not differ significantly (N = 10, P = 0.407), at a 95% CL.

The Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive target organisms in secondary effluent showed that the proportions of the *E. coli* did not differ significantly (N = 10, P = 0.107), the proportions of non-*E. coli* coliforms did not differ significantly (N = 10, P = 0.343), and the proportions of combined coliforms did not differ significantly (N = 10, P = 0.236), all at a 95% CL (Table 3.17).

Table 3.17 - Comparison of Presumptive ESBL and KPC Positive Target Organisms in Morgan Creek, Downstream: Organism-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

***E. coli* Concentration**			**Non-*E. coli* coliforms Concentration**			**Combined Coliforms Concentration**		
N	V	P-value	N	V	P-value	N	V	P-value
10	30	0.107	10	29	0.477	10	30	0.407
% *E. coli*			**% Non-*E. coli* coliforms**			**% Combined Coliforms**		
N	V	P-value	N	V	P-value	N	V	P-value
10	30	0.107	10	31	0.343	10	33	0.236

Jordan Lake

As shown in Table 3.17, the average concentrations of presumptive ESBL and KPC positive *E. coli* in Jordan Lake, downstream of WWTP were 3.50×10^{1} and 8.33×10^{-1} CFU / 100 mL, respectively. The average proportions of ESBL and KPC *E. coli* were 0.48% and 0.67%, respectively. The average concentrations of ESBL and KPC positive other coliforms were 4.02×10^{2} and 5.52×10^{2} CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive other coliforms were 0.84% and 127.15%, respectively. The average concentrations of ESBL and KPC positive combined coliforms were 4.37×10^{2} and 5.53×10^{2} CFU / 100 mL, respectively. The average proportions of ESBL and KPC positive combined coliforms were 1.40% and 153.30%, respectively.

Table 3.18 - Average Concentrations and Proportions of Presumptive ESBL and KPC Positive Target Organisms in Jordan Lake, Downstream of WWTP

Jordan Lake		Bio-Rad Rapid Agar	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
E. coli N = 3	Average	1.42E+02	3.50E+01	8.33E-01	0.48%	0.67%
	Standard Error	6.87E+00	3.42E+00	6.45E-01	0.20%	0.70%
	95% Confidence ±	1.35E+01	6.69E+00	1.27E+00	0.50%	1.30%
Other Coliforms N = 3	Average	2.48E+04	4.02E+02	5.52E+02	0.84%	127.15%
	Standard Error	9.10E+01	1.16E+01	1.66E+01	0.80%	126.50%
	95% Confidence ±	1.78E+02	2.27E+01	3.26E+01	1.60%	248.00%
Combined Coliforms N = 3	Average	2.50E+04	4.37E+02	5.53E+02	0.72%	78.69%
	Standard Error	9.13E+01	1.21E+01	1.66E+01	0.70%	78.20%
	95% Confidence ±	1.79E+02	2.36E+01	3.26E+01	1.40%	153.30%

In Table 3.19, the Wilcoxon Signed Rank comparison of concentrations of presumptive ESBL and KPC positive *E. coli* in Jordan Lake, downstream of WWTP showed that the distribution of the two groups did not differ significantly (N = 3, P = 0.500), at a 95% CL (Table 3.19). The same concentration analysis for the other coliforms and combined coliforms in municipal sewage showed that the non-*E. coli* coliforms

did not differ significantly (N = 3, P = 0.750) and the combined coliform concentrations did not differ significantly (N = 3, P = 0.750), at a 95% CL.

The Wilcoxon Signed Rank tests comparing the proportions of presumptive ESBL and KPC positive target organisms in secondary effluent showed that the proportions of the *E. coli* did not differ significantly (N = 3, P = 1.000), the proportions of non-*E. coli* coliforms did not differ significantly (N = 3, P = 1.000), and the proportions of combined coliforms did not differ significantly (N = 3, P = 0.236), at a 95% CL (Table 3.19).

Table 3.19 - Comparison of Presumptive ESBL and KPC Positive Target Organisms in Jordan Lake: Organism-Specific Wilcoxon Signed Rank Tests of Concentrations and Proportions (α = 0.05)

***E. coli* Concentration**			**Non-*E. coli* coliforms Concentration**			**Combined Coliforms Concentration**		
N	V	P-value	N	V	P-value	N	V	P-value
3	5	0.500	3	4	0.750	3	4	0.750
% *E. coli*			**% Non-*E. coli* coliforms**			**% Combined Coliforms**		
N	V	P-value	N	V	P-value	N	V	P-value
3	3	1.000	3	3	1.000	10	33	0.236

Intra-Site Comparison and Analysis of All Hospital Sites

Kruskal Wallis tests were performed to compare target organism concentration from all the hospital sewage sampling sites (Table 3.20). All tests had a N equal to or greater than 4. No significant differences were found in the concentrations of presumptive *E. coli*, other coliforms, and combined coliforms detected on Bio-Rad and detected on CHROMagar KPC ($P > 0.05$) for all hospital samples, at 95% CI. There were significant differences in the concentrations of presumptive other and combined coliforms directly detected on CHROMagar ESBL ($P = 0.006$ and 0.013, respectively). There was not a significant difference in the ESBL concentration of presumptive *E. coli* ($P = 0.072$).

Table 3.20 - Comparison of Concentrations of Presumptive *E. coli*, Non-*E. coli* coliforms, and Combined coliforms in Hospital Sewage Sites (H1 – H4): Medium- and Organism-Specific Kruskal Wallis Tests (α = 0.05)

Medium	Target Organism	N	CHI SQUARED	DF	P VALUE
Bio-Rad	*E. coli*	4	1.97	3	0.579
	Other coliforms	5	5.18	3	0.159
	Combined coliforms	4	3.19	3	0.364
CHROMagar ESBL	*E. coli*	5	6.99	3	0.072
	Other coliforms	6	12.56	3	**0.006**
	Combined coliforms	5	10.85	3	**0.013**
CHROMagar KPC	*E. coli*	5	3.70	3	0.296
	Other coliforms	6	6.02	3	0.111
	Combined coliforms	5	3.76	3	0.289

Comparison of Raw Sewage and Secondary Effluent

Mann-Whitney U-tests comparing the proportions of presumptive ESBL and KPC positive target organisms in Secondary Effluent and Raw Sewage are shown in Table 3.22. The proportions of the ESBL *E. coli* did not differ significantly (N = 10, P = 0.123), the proportions other coliforms as well as combined coliforms did differ significantly, (N = 10, P = 0.015 and 0.029), at a 95% CL. The proportions of all presumptive KPC positive target organisms did not differ significantly between secondary effluent and raw sewage (N = 10, P > 0.05), at a 95% CL.

3. 21 - Comparison of the Proportions of Presumptive ESBL and KPC Positive Target Organisms in Municipal WWTP Influent and Effluent: Medium-Specific Mann-Whitney U-Tests (α = 0.05)

Resistance Profile	Target Organism	N	W	P VALUE
% ESBL Positive	*E. coli*	10	71	0.123
	Other coliforms	10	82	**0.015**
	Combined coliforms	10	79	**0.028**
% KPC Positive	*E. coli*	10	62	0.393
	Other coliforms	10	28	0.104
	Combined coliforms	10	31	0.162

Comparison of Secondary Effluent and Morgan Creek

Concentrations of presumptive target organisms were compared between Secondary Effluent and Morgan Creek Upstream of the WWTP using Mann-Whitney U-tests (Table 3.23). Test results showed that the concentration distributions between the two sites were significantly different for all target organisms ($P < 0.05$), at a 95% CL.

Table 3.22 – Comparison of Target Organism Concentrations in Secondary Effluent and Morgan Creek Upstream of WWTP: Medium- and Organism-Specific Mann-Whitney U-Tests (α = 0.05)

Medium	Target Organism	N	W	P VALUE
Bio-Rad	*E. coli*	10	97	**0.000**
	Other coliforms	10	88	**0.003**
	Combined coliforms	10	95	**0.000**
CHROMagar ESBL	*E. coli*	10	93	**0.001**
	Other coliforms	10	84	**0.011**
	Combined coliforms	10	92	**0.002**
CHROMagar KPC	*E. coli*	10	85	**0.007**
	Other coliforms	10	86.5	**0.006**
	Combined coliforms	10	86.5	**0.006**

Mann-Whitney U-tests comparing the proportions of presumptive ESBL and KPC positive target organisms in Secondary Effluent and Morgan Creek upstream from the WWTP are shown in Table 3.23. The proportions of all ESBL target organisms in Secondary Effluent did not differ significantly from proportions of all ESBL target organisms in Morgan Creek Upstream of the WWTP ($N = 10$, $P > 0.05$), at a 95% CL. The proportions of the KPC *E. coli* did not differ significantly between Secondary Effluent and Morgan Creek, upstream from its discharge but was borderline ($N = 10$, $P = 0.051$), at the 95% CL. The proportions of the presumptive other KPC positive target organisms in Secondary Effluent did not differ significantly from Morgan Creek, upstream ($N= 10$, $P = >0.05$) at the 95% CL.

Table 3.23 – Comparison of the Proportions of Presumptive ESBL and KPC Positive Target Organisms in Secondary Effluent and Morgan Creek Upstream of WWTP: Medium-Specific Mann-Whitney U-Tests (α = 0.05)

Resistance Profile	Target Organism	N	W	P VALUE
% ESBL Positive	*E. coli*	10	34.5	0.255
	Other coliforms	10	41.5	0.545
	Combined coliforms	10	49	0.971
% KPC Positive	*E. coli*	10	24.5	0.051
	Other coliforms	10	39.5	0.449
	Combined coliforms	10	43	0.623

Concentrations of presumptive target organisms were compared between Secondary Effluent and Morgan Creek Downstream of the WWTP using Mann-Whitney U-tests (Table 3.24). Test results showed that the concentration distributions between the two sites were significantly different for all target organisms ($P < 0.05$), at a 95% CL.

Table 3.24 – Comparison of Target Organism Concentrations in Secondary Effluent and Morgan Creek Downstream of WWTP: Medium- and Organism-Specific Mann-Whitney U-Tests (α = 0.05)

Medium	Target Organism	N	W	P VALUE
Bio-Rad	*E. coli*	10	92	**0.002**
	Other coliforms	10	81	**0.019**
	Combined coliforms	10	88	**0.003**
CHROMagar ESBL	*E. coli*	10	90	**0.003**
	Other coliforms	10	80.5	**0.023**
	Combined coliforms	10	88	**0.005**
CHROMagar KPC	*E. coli*	10	90	**0.002**
	Other coliforms	10	89	**0.004**
	Combined coliforms	10	90	**0.003**

Mann-Whitney U-tests comparing the proportions of presumptive ESBL and KPC positive target organisms in Secondary Effluent and Morgan Creek downstream of the WWTP are shown in Table 3.25. The proportions of all ESBL target organisms in Secondary Effluent did not differ significantly from proportions of all ESBL target organisms in Morgan Creek Upstream of the WWTP (N = 10, P > 0.05), at a 95% CL. However, the proportions of presumptive KPC positive *E. coli* were significantly different (N = 10, P = 0.029), at a 95% CL. The proportions of the presumptive other KPC positive target organisms in Secondary Effluent did not differ significantly from Morgan Creek, upstream (N= 10, P = >0.05) at a 95% CL.

Table 3. 25– Comparison of the Proportions of Presumptive ESBL and KPC Positive Target Organisms in Secondary Effluent and Morgan Creek Downstream of WWTP: Medium-Specific Mann-Whitney U-Tests (α = 0.05)

Resistance Profile	Target Organism	N	W	P VALUE
% ESBL Positive	*E. coli*	10	59.5	0.496
	Other coliforms	10	49.5	1.000
	Combined coliforms	10	54	0.791
% KPC Positive	*E. coli*	10	22	**0.029**
	Other coliforms	10	24.5	0.059
	Combined coliforms	10	29	0.121

Comparison of Morgan Creek Up and Downstream of WWTP

Concentrations of presumptive target organisms were compared between Morgan Creek up and downstream of the WWTP using Mann-Whitney U-tests (Table 3.26). Test results showed that the concentration distributions between the two sites were not significantly different for all target organisms ($P > 0.05$), at a 95% CL.

Table 3.26 – Comparison of Target Organism Concentrations in Morgan Creek Upstream and Downstream of WWTP Discharge: Medium- and Organism-Specific Mann-Whitney U-Tests (α = 0.05)

Medium	Target Organism	N	W	P VALUE
Bio-Rad	*E. coli*	10	49	0.970
	Other coliforms	10	44	0.684
	Combined coliforms	10	44	0.684
CHROMagar ESBL	*E. coli*	10	35	0.265
	Other coliforms	10	40	0.472
	Combined coliforms	10	39	0.427
CHROMagar KPC	*E. coli*	10	56	0.585
	Other coliforms	10	45	0.732
	Combined coliforms	10	45	0.732

Proportions of presumptive ESBL and KPC positive target organisms were compared between Morgan Creek up and downstream of the WWTP using Mann-Whitney U-tests (Table 3.27). Test results showed no significant differences in the proportions all target organisms in the two sites ($P > 0.05$), at a 95% CL.

Table 3.27 – Comparison of the Proportions of Presumptive ESBL and KPC Positive Target Organisms in Morgan Creek Upstream and Downstream of WWTP Discharge: Medium-Specific Mann-Whitney U-Tests (α = 0.05)

Resistance Profile	Target Organism	N	W	P VALUE
% ESBL Positive	*E. coli*	10	33	0.206
	Other coliforms	10	44.5	0.705
	Combined coliforms	10	44.5	0.705
% KPC Positive	*E. coli*	10	54	0.728
	Other coliforms	10	55	0.732
	Combined coliforms	10	52.5	0.879

Objective 3 Results for Presumptive ESBL *E. coli* and Non-*E. coli* Coliforms

Objective three focused on the performance evaluation of CHROMagar ESBL agar medium for the direct detection and quantification of Extended β-lactam resistant *E. coli* and coliforms in the aforementioned nine sampling sites to determine its utility as a part of the proposed indicator system for the surveillance of antimicrobial resistance in the water, wastewater, and other environmental matrices. Presumptive ESBL positive *E. coli* and other coliform isolates were collected during the first phase of the project. These isolates were saved for future analysis outside of this technical report, however, when more funding was awarded in March, 2016, isolate identity confirmation via MALDI-TOF MS was included in the fourth phase of the project. In April, more funding became available that supported VITEK 2 analysis of susceptibility to Cefpodoxime, an Extended-β-lactam, and Imipenem, a carbapenem against associated CLSI MIC criteria. VITEK 2 was also used to evaluate ESBL production in several of the isolates.

Hospital Sewage Presumptive ESBL E. coli and Non-E. coli Isolates

In Table 3.28 are shown number of ESBL isolates from Hospital Sewage chosen for analysis by presumptive identity and then summarizes the number of isolates subjected to confirmatory analysis. A breakdown of the number of isolates in each type of analysis by site and presumptive organism can be found in Tables 2.7.

Table 3.28 - Summary of ESBL Hospital Sewage Isolates and Confirmatory Analyses

Hospital Sewage ESBL Isolates	**Number of Isolates**	**% of Total**
Total Isolates	104	
Presumptive *E. coli* Isolates	35	33.7%
Presumptive other coliform Isolates	69	66.3%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed MALDI-TOF MS	104	100%
Performed VITEK AST - Cefpodoxime Resistance	42	40.4%
Performed VITEK AST - Imipenem Resistance	44	42.3%
Combined MALDI-TOF and VITEK	44	42.3%

In Table 3.29 are shown the results for the 104 ESBL isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual interpretation of colony color, prior to isolation in the original assay. Of the 35 *E. coli* isolates, 5 (14.3%) were positively confirmed as *E. coli* . Twenty-three (65.7%) of presumptive *E. coli* isolates were identified as other coliforms, 7 (20%) were other Gram-Negative bacteria, and 0 were Gram-positive. Sixty-six (95.7%) of the 69 presumptive other coliform isolates were positively confirmed as coliforms. The remaining 3 isolates were other Gram-negative bacteria. Of the 104 total isolates, 4.8% (N = 5) were *E. coli,* 85.6% (N = 89) were Non-*E. coli* coliforms, 9.6% (N = 10) were other Gram-negative bacteria, and 0 were Gram-positive bacteria. See Appendix 3 for a complete results section of MALDI-TOF speciation for all 104 ESBL isolates.

Table 3.29 - MALDI-TOF MS Confirmatory Analysis of Presumptive ESBL *E. coli* and Other Coliform Isolates from Hospital Sewage

Hospital Sewage Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	35	69	104	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	5	66	71	
% Correctly Confirmed	14.3%	95.7%	68.3%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	5	0	5	4.8%
Other Coliforms	23	66	89	85.6%
Other Gram-Negative	7	3	10	9.6%
Gram-Positive / Other	0	0	0	0.0%

In Table 3.30 are shown paired MALDI-TOF and VITEK 2 results for susceptibility to Cefpodoxime and Imipenem in 44 ESBL isolates. Of the 4 confirmed *E. coli* isolates analyzed for resistance to Cefpodoxime, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. Of the 4 confirmed *E. coli* isolates analyzed for resistance to Imipenem, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. Of the 36 confirmed Non-*E. coli*

coliform isolates analyzed for resistance to Cefpodoxime, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. Of the 38 confirmed Non-*E.coli* coliform isolates analyzed for resistance to Imipenem, 39.5% were found to be resistant, 7.9% had intermediate susceptibility, and 52.6% were susceptible. Of the 2 confirmed other Gram-negative bacteria isolates analyzed for resistance to Imipenem, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. See Appendix 3 for a complete results section of MALDI-TOF and VITEK 2 results for all ESBL isolates.

Table 3.30 - VITEK 2 Cefpodoxime and Imipenem Susceptibility Analysis Based on MALDI-TOF MS Identities of Presumptive ESBL *E. coli* and non-*E coli* Isolates from Hospital Sewage

MALDI-TOF MS + VITEK 2	(N)	Number Cefpodoxime Resistant	Number Cefpodoxime Intermediate	Number Cefpodoxime Susceptible	% Res	% Inter	% Suscep
E. coli	4	4	0	0	100%	0%	0%
Other coliforms	36	36	0	0	100.0 %	0.0%	0.0%
Other Gram-Negative bacteria	2	2	0	0	100.0 %	0.0%	0.0%
Gram-Positive Bacteria	0	0	0	0	0%	0.0%	0.0%
Total (N)	42	42	0	0	100.0 %	0.0%	0.0%
MALDI-TOF MS + VITEK 2	**(N)**	**Number Imipenem Resistant**	**Number Imipenem Intermediate**	**Number Imipenem Susceptible**	**% Res**	**% Inter**	**% Suscep**
E. coli	4	4	0	0	100%	0%	0%
Other coliforms	38	15	3	20	39.5%	7.9%	52.6%
Other Gram-Negative bacteria	2	1	1	1	50.0%	50.0%	50.0%
Gram-Positive Bacteria	0	0	0	0	0%	0%	0%
Total (N)	44	20	4	21	45.5%	9.1%	47.7%

Raw Sewage

In Table 3.31 are show the number of ESBL isolates from Raw Sewage chosen for analysis by presumptive identity and then summarizes the number of isolates subjected to confirmatory analysis. A breakdown of the number of isolates in each type of analysis by site and presumptive organism can be found in Tables 2.7.

Table 3.31 - MALDI-TOF MS Confirmatory Analysis of Presumptive ESBL *E. coli* and Other Coliform Isolates from Raw Sewage

Raw Sewage ESBL Isolates	**Number of Isolates**	**% of Total**
Total Isolates	61	
Presumptive *E. coli* Isolates	20	32.8%
Presumptive other coliform Isolates	41	67.2%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed MALDI-TOF MS	61	100.0%
Performed VITEK AST - Cefpodoxime Resistance	56	91.8%
Performed VITEK AST - Imipenem Resistance	57	93.4%
Combined MALDI-TOF and VITEK	57	93.4%

In Table 3.32 are shown the results for the 61 presumptive ESBL *E. coli* and Non-*E. coli* isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual interpretation of colony color, prior to isolation in the original assay. Of the 20 *E. coli* isolates, 14 (70%) were positively confirmed as *E. coli.* Three (15%) of the presumptive *E. coli* isolates were identified as other coliforms, 1 (5%) was another Gram-Negative bacteria and 2 were Gram-positive. Thirty (73.2%) of the 41 presumptive ESBL other coliform isolates were positively confirmed as coliforms. One isolate was *E. coli* and the remaining 10 isolates were other Gram-negative bacteria. Of the 61 total isolates, 24.6% (N = 15) were *E. coli,* 54.1% (N = 33) were Non-*E. coli* coliforms, 18% (N = 11) were other Gram-negative bacteria, and 3.3% (N = 2) were Gram-positive bacteria. See Appendix 3 for a complete results section of MALDI-TOF speciation for all 61 ESBL isolates.

Table 3.32 - MALDI-TOF MS Confirmatory Analysis of Presumptive ESBL *E. coli* and Other Coliform Isolates from Raw Sewage

Raw Sewage Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	20	41	61	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	14	30	44	
% Correctly Confirmed	70.0%	73.2%	72.1%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	14	1	15	24.6%
Other Coliforms	3	30	33	54.1%
Other Gram-Negative	1	10	11	18.0%
Gram-Positive / Other	2	0	2	3.3%

In Table 3.33 are shown paired MALDI-TOF and VITEK 2 results for susceptibility to Cefpodoxime and Imipenem in 57 ESBL isolates. Of the 14 confirmed *E. coli* isolates analyzed for resistance to Cefpodoxime, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. Of the 14 confirmed *E. coli* isolates analyzed for resistance to Imipenem, 93% were found to be resistant, 0 had intermediate susceptibility, and 7% were susceptible. Of the 32 confirmed Non-*E. coli* coliform isolates analyzed for resistance to Cefpodoxime, 96.9% were found to be resistant, 3.1% had intermediate susceptibility, and 0 were susceptible. Of the 33 confirmed Non-*E. coli* coliform isolates analyzed for resistance to Imipenem, 72.7% were found to be resistant, 15.2% had intermediate susceptibility, and 12.1% were susceptible. Of the 10 confirmed other Gram-negative bacteria isolates analyzed for resistance to Imipenem, 80% were found to be resistant, 0 had intermediate susceptibility, and 20% were susceptible. See Appendix 3 for a complete results section of MALDI-TOF and VITEK 2 results for all 57 ESBL isolates.

Table 3.33 - Table 3.30 - VITEK 2 Cefpodoxime and Imipenem Susceptibility Analysis Based on MALDI-TOF MS Identities of Presumptive ESBL E. coli and non-E. coli Isolates from Raw Sewage

MALDI-TOF MS + VITEK 2	(N)	Number Cefpodoxime Resistant	Number Cefpodoxime Intermediate	Number Cefpodoxime Susceptible	% Res	% Inter	% Suscep
E. coli	14	14	0	0	100%	0%	0%
Other coliforms	32	31	1	0	96.9%	3.1%	0.0%
Other Gram-Negative bacteria	10	8	0	2	80.0%	0.0%	20.0%
Gram-Positive Bacteria	0	0	0	0	0%	0%	0%
Total (N)	56	53	1	0	94.6%	1.8%	0.0%
MALDI-TOF MS + VITEK 2	**(N)**	**Number Imipenem Resistant**	**Number Imipenem Intermediate**	**Number Imipenem Susceptible**	**% Res**	**% Inter**	**% Suscep**
E. coli	14	13	0	1	93%	0%	7%
Other coliforms	33	24	5	4	72.7%	15.2%	12.1%
Other Gram-Negative bacteria	10	1	3	6	10.0%	30.0%	60.0%
Gram-Positive Bacteria	0	0	0	0	0%	0%	0%
Total (N)	57	38	8	11	66.7%	14.0%	19.3%

Secondary Effluent

In Table 3.34 are show the number of presumptive ESBL *E. coli* and non-*E. coli* isolates from Secondary Effluent chosen for analysis by presumptive identity and then summarizes the number of isolates subjected to confirmatory analysis. A breakdown of the number of isolates in each type of analysis by site and presumptive organism can be found in Tables 2.7.

Table 3.34 - Summary of ESBL Secondary Effluent Isolates and Confirmatory Analyses

Secondary Effluent ESBL Isolates	**Number of Isolates**	**% of Total**
Total Isolates	64	
Presumptive *E. coli* Isolates	29	45.3%
Presumptive other coliform Isolates	35	54.7%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed MALDI-TOF MS	64	100.0%
Performed VITEK AST - Cefpodoxime Resistance	57	91.8%
Performed VITEK AST - Imipenem Resistance	60	93.4%
Combined MALDI-TOF and VITEK	57	93.4%

In Table 3.35 are shown the results for the 60 ESBL isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual interpretation of colony color, prior to isolation in the original assay. Of the 27 *E. coli* isolates, 23 (85.2%) were positively confirmed as *E. coli*. Three (11.1%) of the presumptive *E. coli* isolates were identified as other coliforms, 1 (3.7%) was another Gram-Negative bacteria, and none were Gram-positive. Twenty-six (78.8%) of the 33 presumptive other coliform isolates were positively confirmed as coliforms. One isolate was *E. coli*, and the remaining 6 isolates were other Gram-negative bacteria. Of the 60 total isolates, 40% (N = 24) were *E. coli,* 48.3% (N = 29) were Non-*E. coli* coliforms, 11.7% (N = 7) were other Gram-negative bacteria, and 0 were Gram-positive bacteria. See Appendix 3 for a complete results section of MALDI-TOF speciation for all 60 ESBL isolates.

Table 3.35 - MALDI-TOF MS Confirmatory Analysis of Presumptive ESBL *E. coli* and Other Coliform Isolates from Secondary Effluent

Secondary Effluent Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	27	33	60	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	23	26	49	
% Correctly Confirmed	85.2%	78.8%	81.7%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	23	1	24	40.0%
Other Coliforms	3	26	29	48.3%
Other Gram-Negative	1	6	7	11.7%
Gram-Positive / Other	0	0	0	0.0%

Table 3.36 shows paired MALDI-TOF and VITEK 2 results for susceptibility to Cefpodoxime and Imipenem in 60 ESBL isolates. Of the 24 confirmed *E. coli* isolates analyzed for resistance to Cefpodoxime, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. Of the 24 confirmed *E. coli* isolates analyzed for resistance to Imipenem, 100% were found to be resistant, 0 had intermediate susceptibility, and 0 were susceptible. Of the 26 confirmed Non-*E. coli* coliform isolates analyzed for resistance to Cefpodoxime, 96.2% were found to be resistant, 3.8% had intermediate susceptibility, and 0 were susceptible. Of the 29 confirmed Non-*E. coli* coliform isolates analyzed for resistance to Imipenem, 75.9% were found to be resistant, 6.9% had intermediate susceptibility, and 17.2% were susceptible. Of the 7 confirmed other Gram-negative bacteria isolates analyzed for resistance to Imipenem, 14.3% were found to be resistant, 42.9% had intermediate susceptibility, and 42.9% were susceptible. See Appendix 3 for a complete results section of MALDI-TOF and VITEK 2 results for all 60 ESBL isolates.

Table 3.36 - *VITEK 2* Cefpodoxime and Imipenem Susceptibility Analysis Based on MALDI-TOF MS Identities of Presumptive ESBL *E. coli and non-E coli* Coliform Isolates from Secondary Effluent

MALDI-TOF MS + VITEK 2	(N)	Number Cefpodoxime Resistant	Number Cefpodoxime Intermediate	Number Cefpodoxime Susceptible	% Res	% Inter	% Suscep
E. coli	24	24	0	0	100%	0%	0%
Other coliforms	26	25	1	0	96.2%	3.8%	0.0%
Other Gram-Negative bacteria	7	7	0	0	100.0 %	0.0%	0.0%
Gram-Positive Bacteria	0	0	0	0	0%	0%	0%
Total (N)	57	56	1	0	98.2%	1.8%	0.0%
MALDI-TOF MS + VITEK 2	**(N)**	**Number Imipenem Resistant**	**Number Imipenem Intermediate**	**Number Imipenem Susceptible**	**% Res**	**% Inter**	**% Suscep**
E. coli	24	24	0	0	100%	0%	0%
Other coliforms	29	22	2	5	75.9%	6.9%	17.2%
Other Gram-Negative bacteria	7	1	3	3	14.3%	42.9%	42.9%
Gram-Positive Bacteria	0	0	0	0	0%	0%	0%
Total (N)	60	47	5	8	78.3%	8.3%	13.3%

Objective 4 Results for Presumptive Carbapenem (KPC) Resistant *E. coli* and Non-*E. coli* Coliforms

Objective four focused on the performance evaluation of CHROMagar KPC agar medium for the direct detection and quantification of carbapenemase producing (KPC) *E. coli* and non-*E. coli* coliforms in the aforementioned nine sampling sites to determine its utility as a part of the proposed indicator system for the surveillance of antimicrobial resistance in the water, wastewater, and other environmental matrices. Presumptive carbapenemase-positive *E. coli* and other non-*E. coli* coliform isolates were collected during the first phase of the project. Phase 3 focused on the revival, purification, and non-automated, antimicrobial susceptibility analysis of carbapenemase production using meropenem disks in the Modified Hodge Test. Phase 4 focused on isolate identity confirmation of selected isolates via MALDI-TOF MS (Table 2.8). Several of the surface water isolates and a few of hospital sewage and secondary effluent isolates were also subjected to VITEK 2 analysis for susceptibility to Cefpodoxime, an Extended-β-lactam, and Imipenem, a carbapenem, against associated CLSI MIC criteria (Table 2.9).

Of the 354 presumptive KPC positive isolates collected, 300 were successfully revived (84.7%). Analysis of KPC production via the Modified Hodge Test (N=298) occurred before a portion of the isolates were subjected to identity confirmation via MALDI-TOF MS (N = 233). After both Modified Hodge and MALDI-TOF analysis, 23 isolates were selected for analysis via VITEK-2 for further confirmation and analysis

CHROMagar KPC Performance Analysis for Presumptive KPC E. coli and Non-E. coli Isolates from Hospital Sewage

In Table 3.37 are shown the number of successfully revived KPC isolates from Hospital Sewage by presumptive identity as *E. coli* or non-*E. coli* coliforms and then a summary of the number of isolates subjected to confirmatory analysis. A breakdown of the number of presumptive isolates in each type of analysis by site and presumptive organism can be found in Tables 2.8 and 2.9.

Table 3.37 - Summary of KPC Hospital Sewage Isolates and Confirmatory Analyses

Hospital Sewage KPC Isolates	**Number of Isolates**	**% of Total**
Total Isolates	166	
Presumptive *E. coli* Isolates	52	31.3%
Presumptive other coliform Isolates	114	68.7%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed Modified Hodge Test	164	98.8%
Performed MALDI-TOF MS	112	67.5%
Combined MALDI-TOF and Modified Hodge	110	66.3%
Combined MALDI-TOF, Modified Hodge, and VITEK 2	4	2.4%

Of the 52 hospital sewage isolates of presumptive KPC positive *E. coli* subjected to analysis via the Modified Hodge Test, 12 (23.1%) were positive for KPC production, 31 (59.6%) showed signs of "intermediate" KPC production, and 8 (17.3%) were negative (Table 3.38). Site H1 had the highest proportion of positive results (66.7%, N = 9). Analysis of the 112 hospital sewage other coliform isolates showed more KPC production overall, with 68 (60.75%) positive results, 28 (25%) intermediate, and 15 (13.4%) negative results. Site H1 also had the highest proportion of positive results overall (93.3%, N = 45).

Table 3.38 - Modified Hodge Test Results for Presumptive KPC Hospital Sewage Isolates

KPC Hospital Sewage	Modified Hodge Test (N)	KPC(+)	%	KPC(-R)	%	KPC(-)	%
E. coli							
OE H1 GI + MICU	9	6	66.7%	2	22.2%	1	11.1%
PE H2 Burn + Lab + ICU	17	1	5.9%	13	76.5%	3	17.6%
QE H3 Heart + ICU	11	0	0.0%	8	72.7%	3	27.3%
RE H4 ER + Neuro	15	5	33.3%	8	53.3%	2	13.3%
Total	**52**	**12**	**23.1%**	**31**	**59.6%**	**8**	**17.3%**
KPC Hospital Sewage Isolates	**Modified Hodge Test (N)**	**KPC(+)**	**%**	**KPC(-R)**	**%**	**KPC(-)**	**%**
Non-*E. coli* coliforms							
OT H1 GI + MICU	45	42	93.3%	2	4.4%	1	2.2%
PT H2 Burn + Lab + ICU	27	9	33.3%	10	37.0%	7	25.9%
QT H3 Heart + ICU	16	5	31.3%	10	62.5%	1	6.3%
RT H4 ER + Neuro	24	12	50.0%	6	25.0%	6	25.0%
Total	**112**	**68**	**60.7%**	**28**	**25.0%**	**15**	**13.4%**

In Table 3.39 are shown the results for the 112 presumptive KPC isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual interpretation of colony color, prior to isolation in the original assay. One of the 16 *E. coli* isolates was positively confirmed as *E. coli*. Ten (6.3%) of the presumptive *E. coli* isolates were identified as other coliforms, 8 (50%) were other Gram-Negative bacteria such as *Aeromonas, Cronobacter, Raoutella,* and *Stenotrophomonas,* and none were Gram-positive. Eighty-nine (92.7%) of the 96 presumptive other coliform isolates were positively confirmed as coliforms. The remaining isolates were identified as 6 other Gram-negative bacteria *Ochrobactrum, Stenotrophomonas* and *Cronobacter*) and 1 Gram-positive bacterium. Of the 112 total isolates, 0.8% (N = 1) were *E. coli,* 86.6% (N = 97) were Non-*E. coli* coliforms, 11.6% (N = 13) were other Gram-negative bacteria, and 0.9% (N = 1) were Gram-positive bacteria. See Appendix 4 for a complete results section of MALDI-TOF speciation for all 112 KPC hospital sewage isolates.

Table 3.39 - MALDI-TOF MS Confirmatory Analysis of Presumptive KPC *E. coli* and Other Coliform Isolates from Hospital Sewage

Raw Sewage Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	16	96	112	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	1	89	90	
% Correctly Confirmed	6.3%	92.7%	80.4%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	1	0	1	0.9%
Other Coliforms	8	89	97	86.6%
Other Gram-Negative	7	6	13	11.6%
Gram-Positive / Other	0	1	1	0.9%

In Table 3.40 are shown paired MALDI-TOF and Modified Hodge Test results for 110 hospital sewage isolates. Of the 1 confirmed *E. coli* isolate, it was negative for KPC production and had intermediate production. Of the 95 confirmed as Non-*E. coli* coliform isolates, 95 (61.5%) were positive for KPC production, 63 (21.1%) had intermediate production, and 11 (11.6%) were negative. Of the 13 isolates confirmed as other Gram-Negative bacteria, 2 (15.4%) were positive for KPC production, 8 (61.5%) had intermediate production, and 3 (23.1%) were negative. See Appendix 4 for a complete results section of MALDI-TOF and Modified Hodge results for all 112 KPC hospital sewage isolates.

Table 3.40 - Modified Hodge Test Results of KPC Production Based on MALDI-TOF MS Identities of Isolates from Hospital Sewage

	Total (N)	**KPC(+)**	**KPC(-R)**	**KPC(-)**	**% KPC(+)**	**% KPC(-R)**	**% KPC (-)**
E. coli	1	0	1	0	0%	100%	0%
Other coliforms	95	63	20	11	66.3%	21.1%	11.6%
Other Gram-Negative bacteria	13	2	8	3	15.4%	61.5%	23.1%
Gram-Positive Bacteria	1	0	1	0	0.0%	100.0%	0.0%
Total	110	65	30	14	59.1%	27.3%	12.7%

Four KPC hospital sewage isolates were submitted for VITEK 2 analysis because the isolates grew on the CHROMagar KPC medium but had negative Modified Hodge Test results. Table 3.41 shows the combined results. Complete isolate analysis can be found Appendix 5.

Table 3.41 - KPC Hospital Sewage Isolates with Combined VITEK 2, MALDI-TOF MS, and Modified Hodge Test Results

Code	MALDI-TOF Result	Cefpodoxime	Imipenem	Hodge Results	Confirmation
		S/I/R	S/I/R		
RT04	*Enterobacter cloacae/asburiae*	I	S	KPC(-)	Confirms KPC (-)
RT07	*Raoultella planticola/ornithinolytica*	R	I	KPC(-)	Does not confirm interpretation
RT10	*Raoultella planticola/ornithinolytica*	R	S	KPC(-)	Confirms KPC (-)
RT18	*Citrobacter amalonaticus*	R	S	KPC(-)	Confirms KPC (-)

CHROMagar KPC Performance Analysis for Presumptive KPC E. coli and Non-E. coli Isolates from Raw Sewage

In Table 3.42 are shown the number of successfully revived KPC isolates from raw sewage by presumptive identity and then a summary of the number of isolates subjected to confirmatory analysis. A breakdown of the number of isolates in each type of analysis by presumptive organism can be found in Tables 2.8.

Table 3.42- Summary of KPC Raw Sewage Isolates and Confirmatory Analyses

Raw Sewage KPC Isolates	**Number of Isolates**	**% of Total**
Total Isolates	58	
Presumptive *E. coli* Isolates	16	27.6%
Presumptive other coliform Isolates	42	72.4%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed Modified Hodge Test	58	100.0%
Performed MALDI-TOF MS	54	93.1%
Combined MALDI-TOF and Modified Hodge	54	93.1%

Modified Hodge test results for 16 raw sewage isolates of presumptive KPC positive *E. coli* indicated that 10 (62.5%) were positive for KPC production, 5 (31.1%) had "intermediate" production, and 1 (6.3%) were negative (Table 3.43) The same analysis of 42 presumptive other coliform isolates showed that 34 (81.1%) were positive for KPC production, 4 (9.5%) showed signs of "intermediate" KPC production, and 4 (9.5%) were negative.

Table 3.43 - Modified Hodge Analysis of Presumptive KPC positive *E. coli* and Other Coliform Isolates from Raw Sewage

KPC Municipal Sewage Isolates	**Modified Hodge Test (N)**	**KPC(+)**	**%**	**KPC(-R)**	**%**	**KPC(-)**	**%**
JT Non-*E. coli* coliforms	42	34	81.0%	4	9.5%	4	9.5%
JE *E. coli*	16	10	62.5%	5	31.3%	1	6.3%

In Table 3.44 are shown the results for the 54 raw sewage KPC isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual

interpretation of colony color, prior to isolation in the original assay. None of the 12 presumptive *E. coli* isolates were positively confirmed by MALDI-TOF MS. Eight of the presumptive *E. coli* isolates were identified as other coliforms, 3 were other Gram-Negative bacteria such as *Pseudomonas* and *Stentrophomonas,* and the 1 Gram-positive was *Enterococcus*. Thirty-six (85.7%) of the 42 presumptive other coliform isolates were positively confirmed as coliforms. The remaining 6 isolates were other Gram-negative bacteria including *Cronobacter* and *Aeromonas.* Of the 54 total isolates, 81.5% (N = 44) were Non-*E. coli* coliforms, 16.7% (N = 9) were other Gram-negative bacteria, and 1.9% (N = 1) was a Gram-positive bacterium. See Appendix 4 for a complete results section of MALDI-TOF speciation for all 54 raw sewage isolates.

Table 3.44 - MALDI-TOF MS Confirmatory Analysis of Presumptive KPC *E. coli* and Other Coliform Isolates from Raw Sewage

Raw Sewage Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	12	42	54	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	0	36	36	
% Correctly Confirmed	0.0%	85.7%	66.7%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	0	0	0	0.0%
Other Coliforms	8	36	44	81.5%
Other Gram-Negative	3	6	9	16.7%
Gram-Positive / Other	1	0	1	1.9%

In Table 3.45 are shown paired MALDI-TOF and Modified Hodge Test results for 54 KPC raw sewage isolates. Of the 44 confirmed coliform isolates, 81.8% were positive for KPC production, 11.4% had intermediate production, and 6.8% were negative. Of the 9 isolates confirmed as other Gram-Negative bacteria, 5 were positive for KPC production, 3 had intermediate production, and 1 was negative. See Appendix 4 for a complete results section of MALDI-TOF and Modified Hodge results for all 54 raw sewage KPC isolates.

Table 3.45 - Modified Hodge Test Results of KPC Production Based on MALDI-TOF MS Identities of Isolates from Raw Sewage

	Total (N)	KPC(+)	KPC(-R)	KPC(-)	% KPC(+)	% KPC(-R)	% KPC (-)
E. coli	0	0	1	0	0.0%	0.0%	0.0%
Other coliforms	44	36	5	3	81.8%	11.4%	6.8%
Other Gram-Negative bacteria	9	5	3	1	55.6%	33.3%	11.1%
Gram-Positive Bacteria	1	0	0	1	0.0%	0.0%	100.0%
Total	54	41	9	5	75.9%	16.7%	9.3%

CHROMagar KPC Performance Analysis for Presumptive KPC E. coli and Non-E. coli Isolates from Secondary Effluent

In Table 3.46 are shown numbers of successfully revived presumptive KPC isolates from secondary effluent by presumptive identity and then a summary of the number of isolates subjected to confirmatory analysis. A breakdown number of isolates in each type of analysis by presumptive organism can be found in Tables 2.8 and 2.9.

Table 3.46 - Summary of Presumptive KPC Secondary Effluent Isolates and Confirmatory Analyses

Surface Water Isolates	**Number of Isolates**	**% of Total**
Total Isolates	49	
Presumptive *E. coli* Isolates	17	34.7%
Presumptive other coliform Isolates	32	65.3%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed Modified Hodge Test	49	100.0%
Performed MALDI-TOF MS	38	77.6%
Combined MALDI-TOF and Modified Hodge	38	77.6%
Combined MALDI-TOF, Modified Hodge, and VITEK 2	4	8.2%

In Table 3.47 are shown Modified Hodge test results for 17 secondary effluent isolates of presumptive KPC positive *E. coli* with 23 (76.5%) positive for KPC production and 4 (23.5%) "intermediate" production. The same analysis of 32 other coliform isolates showed that 23 (71.9%) were positive for KPC production, 5 (15.6%) showed signs of "intermediate" KPC production, and 4 (12.5%) were negative.

Table 3.47 - Modified Hodge Test Results for KPC Secondary Effluent Isolates

KPC Treated Sewage Isolates		**Modified Hodge Test (N)**	**KPC(+)**	**%**	**KPC(-R)**	**%**	**KPC(-)**	**%**
KT	Non-*E. coli* coliforms	32	23	71.9%	5	15.6%	4	12.5%
KE	*E. coli*	17	13	76.5%	4	23.5%	0	0.0%

In Table 3.48 are shown the results for 38 secondary effluent KPC isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual interpretation of colony color, prior to isolation in the original assay. None of the 16 *E. coli* isolates were positively confirmed as *E. coli*. Ten (62.5%) of the presumptive *E. coli* isolates were identified as other coliforms, 6 (37.5%) were other Gram-Negative bacteria such as *Pseudomonas* and *Stenotrophomonas,* and none were Gram-positive. Twenty (91%) of the 22 presumptive other coliform isolates were positively confirmed as coliforms. One remaining isolate was *E. coli* and 1 remaining isolate was another Gram-negative bacterium, *Pantoea agglomerans*. Of the 38 total presumptive other KPC isolates, 2.6% (N = 1) were *E. coli,* 78.9% (N = 30) were Non-*E. coli* coliforms, 18.4% (N = 7) were other Gram-negative bacteria, and 0% (N = 0) were Gram-positive bacteria. See Appendix 4 for a complete results section of MALDI-TOF speciation for all 38 secondary effluent KPC isolates.

Table 3.48 - MALDI-TOF MS Confirmatory Analysis of Presumptive KPC *E. coli* and Other Coliform Isolates from Secondary Effluent

Raw Sewage Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	16	22	38	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	0	20	20	
% Correctly Confirmed	0.0%	90.9%	52.6%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	0	1	1	2.6%
Other Coliforms	10	20	30	78.9%
Other Gram-Negative	6	1	7	18.4%
Gram-Positive / Other	0	0	0	0.0%

In Table 3.49 are shown paired MALDI-TOF and Modified Hodge Test results for 38 secondary effluent KPC isolates. Of the 1 confirmed *E. coli* isolate was found to be negative for KPC production. Of the 30 confirmed as Non-*E. coli* coliform isolates, 80% were positive for KPC production, 10% had intermediate production, and 10% were negative. Of the 7 isolates confirmed as other Gram-Negative bacteria, 57.1% were positive for KPC production, 42.9% had intermediate production, and 0 were

negative. See Appendix 4 for a complete results section of MALDI-TOF and Modified Hodge results for all 38 secondary effluent KPC isolates.

Table 3.49 - Modified Hodge Test Results of KPC Production Based on MALDI-TOF MS Identities of Presumptive KPC Isolates from Secondary Effluent

	Total (N)	KPC(+)	KPC(-R)	KPC(-)	% KPC(+)	% KPC(-R)	% KPC (-)
E. coli	1	0	0	1	0.0%	0.0%	100%
Other coliforms	30	24	3	3	80.0%	10.0%	10.0%
Other Gram-Negative bacteria	7	4	3	0	57.1%	42.9%	0.0%
Gram-Positive Bacteria	0	0	0	0	0.0%	0.0%	0.0%
Total	38	28	6	4	73.7%	15.8%	10.5%

Four KPC secondary effluent isolates were submitted for VITEK 2 analysis because all isolates were identified as target organisms via MALDI-TOF (Table 3.50). One of them (KT16) was originally detected on CHROMagar KPC, but, after revival in TSB and on CHROMagar Orientation, it did not grow on the CHROMagar KPC medium and had negative Modified Hodge Test results. VITEK 2 confirmed susceptibility to both Cefpodoxime and Imipenem. The other three isolates were chosen to evaluate the "intermediate KPC production" designation created by this study. All three were resistant to Cefpodoxime. One isolate (KT24) was resistant to Imipenem and the other two (KT35A and B) had intermediate resistance when compared to CLSI MIC criteria. Complete isolate analysis can be found Appendix 5.

Table 3.50 - Secondary Effluent Presumptive KPC Isolates with Combined VITEK 2, MALDI-TOF MS, and Modified Hodge Test Results

Code	MALDI-TOF Result	Cefpodoxime	Imipenem	Hodge Results	Confirmation
		S/I/R	S/I/R		
KT16	*Escherichia coli*	S	S	KPC(-)	Confirms KPC (-)
KT24	*Klebsiella pneumoniae*	R*	R*	KPC(-R)	Confirms KPC (-R)
KT35A	*Enterobacter cloacae/asburiae*	R	I	KPC(-R)	Confirms KPC(-R)
KT35B	*Enterobacter cloacae/asburiae*	R	I	KPC(-R)	Confirms KPC(-R)

CHROMagar KPC Performance Analysis for Presumptive KPC E. coli and Non-E. coli Isolates from Surface Water

In Table 3.51 are shown the number of successfully revived KPC isolates from Surface Water by presumptive identity and then a summary of the number of isolates subjected to confirmatory analysis. A breakdown of the number of isolates in each type of analysis by site and presumptive organism can be found in Tables 2.8 and 2.9.

Table 3.51 - Summary of KPC Surface Water Isolates and Confirmatory Analyses

Surface Water Isolates	**Number of Isolates**	**% of Total**
Presumptive other coliform Isolates	29	100%
Summary of Analyses	**Number of Isolates**	**% of Total**
Performed Modified Hodge Test	28	96.5%
Performed MALDI-TOF MS	29	100.0%
Combined MALDI-TOF and Modified Hodge	28	96.5%
Combined MALDI-TOF, Modified Hodge, and VITEK 2	22	75.9%

Of the 27 surface water isolates of presumptive KPC positive other coliforms subjected to analysis via the Modified Hodge Test, zero were positive for KPC production, 19 (70.4%) showed signs of "intermediate" KPC production, and 8 (29.6%) were negative (Table 3.52). Morgan Creek, upstream had the highest proportion of intermediate production (75.0%, N = 8). There were no KPC positive *E. coli* isolates collected or analyzed during this project.

Table 3.52 - Modified Hodge Test Results for KPC Surface Water Isolates

KPC Surface Water Isolates		**Modified Hodge Test (N)**	**KPC(+)**	**%**	**KPC(-R)**	**%**	**KPC(-)**	**%**
Non-*E. coli* coliforms								
LT	Morgan Creek Upstream	8	0	0.0%	6	75.0%	2	25.0%
MT	Morgan Creek Downstream	11	0	0.0%	8	72.7%	3	27.3%
NT	Jordan Lake	8	0	0.0%	5	62.5%	3	37.5%
	Total	**27**	**0**	**0.0%**	**19**	**70.4%**	**8**	**29.6%**

In Table 3.53 are shown the results for the 28 presumptive KPC other coliform isolates that were subjected to MALDI-TOF MS for confirmation of presumptive identities that were based on the original visual interpretation of colony color, prior to isolation in the original assay. There were no presumptive *E. coli* isolates. 0 of the 28 presumptive other coliform isolates were positively confirmed as coliforms. 26 isolates were other Gram-negative bacteria including *Ochrobactrum, Sphingebacterium,* and *Stenotrophomonas*, and 2 were Gram-Positive *Staphlococcus* and *Bacillius.* Of the 28 presumptive KPC total isolates, 0 were *E. coli,* 0 were Non-*E. coli* coliforms, 89.7% (N = 26) were other Gram-negative bacteria, and 6.9% (N = 2) were Gram-positive bacteria. See Appendix 4 for a complete results section of MALDI-TOF speciation for all 28 surface water KPC isolates.

Table 3.53 - MALDI-TOF MS Confirmatory Analysis of Presumptive KPC *E. coli* and Other Coliform Isolates from Surface Water

Raw Sewage Isolates	**Presumptive *E. coli***	**Presumptive coliform**	**Total Isolates**	
Original Number of Isolates	0	28	28	
Number of Correctly Confirmed based on original presumptive ID prior to isolation	0	0	0	
% Correctly Confirmed	0.0%	0.0%	0.0%	
MALDI-TOF Identity	**Presumptive *E. coli***	**Presumptive coliform**	**# Isolates**	**% Total**
E. coli	0	0	0	0.0%
Other Coliforms	0	0	0	0.0%
Other Gram-Negative	0	26	26	89.7%
Gram-Positive / Other	0	2	2	6.9%

In Table 3.54 are shown paired MALDI-TOF and Modified Hodge Test results for 25 surface water KPC isolates. There were no confirmed *E. coli* isolates. There were no confirmed Non-*E. coli* coliform isolates. Of the 25 isolates confirmed as other Gram-Negative bacteria, 0 were positive for KPC production, 56% had intermediate production, and 44% were negative. See Appendix 4 for a complete results section of MALDI-TOF and Modified Hodge results for all 25 surface KPC isolates.

Table 3.54 - Modified Hodge Test Results of KPC Production Based on MALDI-TOF MS Identities of Isolates From Surface Water

	Total (N)	KPC(+)	KPC(-R)	KPC(-)	% KPC(+)	% KPC(-R)	% KPC (-)
E. coli	0	0	0	0	0.0%	0.0%	0.0%
Other coliforms	0	0	0	0	0.0%	0.0%	0.0%
Other Gram-Negative bacteria	25	0	14	11	0.0%	56.0%	44.0%
Gram-Positive Bacteria	0	0	0	1	0.0%	0.0%	0.0%
Total	25	0	14	12	0.0%	56.0%	48.0%

Twenty-two KPC surface water isolates were submitted for VITEK 2 analysis, however, those identified as *Stenotrophomonas maltophilia* via MALDI-TOF MS were rejected by the VITEK 2 system due to lack of information in the API database. Table 3.55 is the results for the remaining 17 isolates. None of the isolates were identified as target organisms, but grew on CHROMagar KPC medium. Eleven isolates had the "intermediate KPC production" designation created by this study. Of these, six were found to be resistant to Imipenem and five were found to be susceptible. Nine of the eleven were resistant to Cefpodoxime and two had intermediate susceptibility. Six of the isolates had negative Modified Hodge test results. Of these 6, five were susceptible to Imipenem and one was resistant. Furthermore, five of these KPC(-) isolates were resistant to Cefpodoxime, one was susceptible, and one had intermediate resistance. Complete isolate analysis can be found Appendix 5.

Table 3.55 - Surface Water Isolates with Combined VITEK 2, MALDI-TOF MS, and Modified Hodge Test Results

Code	Site	*MALDI-TOF Result*	Cefpodoxime	Imipenem	Hodge Results	Confirmation
			S/I/R	S/I/R		
LT02	Morgan Creek Upstream	*Ochrobactrum anthropic*	R	S	KPC(-R)	
LT04	Morgan Creek Upstream	*Ochrobactrum anthropic*	R	S	KPC(-R)	
LT05	Morgan Creek Upstream	*Ochrobactrum anthropic*	I	S	KPC(-R)	
LT07	Morgan Creek Upstream	*Ochrobactrum anthropic*	R	S	KPC(-R)	
LT10	Morgan Creek Upstream	*Sphingobacterium multivorum*	R	R	KPC(-)	
LT11	Morgan Creek Upstream	*Sphingobacterium multivorum*	R	R	KPC(-R)	Confirms KPC(-R)
MT04	Morgan Creek Downstream	*Pseudomonas chororaphis/fluorescens*	R	R	KPC(-R)	Confirms KPC(-R)
MT05	Morgan Creek Downstream	*Ochrobactrum anthropic*	R	S	KPC(-)	Confirms KPC (-)
MT06	Morgan Creek Downstream	*Ochrobactrum anthropic*	R	S	KPC(-R)	
MT07	Morgan Creek Downstream	*Ochrobactrum anthropic*	R	S	KPC(-)	Confirms KPC (-)
MT09	Morgan Creek Downstream	*Ochrobactrum anthropic*	R	S	KPC(-)	Confirms KPC (-)
MT11	Morgan Creek Downstream	*Elizabethkingia meningoseptica*	R	R	KPC(-R)	Confirms KPC(-R)
MT13	Morgan Creek Downstream	*Sphingobacterium multivorum*	I	R	KPC(-R)	Confirms KPC(-R)
NT02	Jordan Lake	*Sphingobacterium multivorum*	R	R	KPC(-R)	Confirms KPC(-R)
NT05	Jordan Lake	*Sphingobacterium multivorum*	R	R	KPC(-R)	Confirms KPC(-R)
NT06	Jordan Lake	*Ochrobactrum anthropic*	S	S	KPC(-)	Confirms KPC (-)
NT07	Jordan Lake	*Ralstonia insidiosa*	I	S	KPC(-)	Confirms KPC (-)

CHAPTER 4 DISCUSSION, RECOMMENDATIONS, AND CONCLUSIONS

Discussion:

Repeated and often parallel assays of representative wastewater and environmental surface water samples were performed using the procedures of EPA Method 1604 for membrane filtration followed by plating on CHROMagar ESBL and KPC agar media. These assays were done in parallel with plating the same samples on the aforementioned Bio-Rad Rapid'*E. coli 2* agar/CHROMagar Orientation agar to determine percentages of all culturable *E. coli* and coliforms that are resistant to these classes of antimicrobials. Following incubation, presumptive colonies of *E. coli* and coliforms were enumerated using manufacturers' guides for colony color and appearance (Table 2.5 and 2.6). During each assay, five or fewer representative colonies of presumptive *E. coli* and other coliforms were selected from the CHROMagar ESBL and KPC membranes. Each colony was presumed to express the resistance property of the culture medium on which they were detected. Presumptive positive colonies were streaked successively for isolation, purification and preservation for future analysis to determine the performance of the two culture media in hospital sewage, raw sewage, secondary effluent and surface waters.

The first objective of this study was to evaluate the performance of a chromogenic substrate standard clinical agar medium for use in the direct detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in representative wastewaters and surface waters by comparing it to the performance of a "gold standard" chromogenic substrate agar medium for the direct detection and enumeration of *E. coli* and coliforms in the same samples.

The results from the Wilcoxon signed-rank test comparing the clinical medium (CHROMagar Orientation) against the performance of the "gold standard" environmental medium (Bio-Rad Rapid'*E. coli 2* agar) for the detection of presumptive *E. coli* was comparable in hospital sewage and treated sewage effluent matrices at a 95% CL (Table 3.1). The clinical medium performance was not comparable

in the detection of presumptive *E. coli* in raw sewage and surface water samples. In both matrices, the clinical medium detected on average 20% more *E. coli* than the environmental medium (Tables 5.A, 7.A, and 8.A in Appendix 2).

The results from the Wilcoxon signed-rank test comparing the clinical medium (CHROMagar Orientation) against the performance of the "gold standard", environmental medium (Bio-Rad Rapid'*E. coli 2* agar) for the detection of presumptive other and combined coliforms were comparable and thus capable for use in the direct detection of these target organisms in hospital sewage, raw sewage, treated sewage effluent, and surface water matrices at a 95% CL (Tables 3.2 and 3.3).

Results from this study suggest that the performance of CHROMagar Orientation in raw sewage and surface water matrices for the detection of *E. coli* do not fulfill the needed requirements for the proposed method associated with discrimination of target bacteria in mixed microbial communities of environmental matrices. CHROMagar Orientation was originally validated for urine and stool analysis, not analysis of environmental samples[86]. The medium is also inherently non-selective, and supports interfering growth and overgrowth of other Gram-negative and Gram-positive bacteria like *Pseudomonas* spp. and *Enterococcus* spp. Limitations in the performance of the clinical medium may also be due to colony visualization and characterization issues by the analyst. Merlino et al. found that the color of bacterial colonies grown on Orientation are stable in the dark, but change when exposed to light, becoming deeper in tone and more diffuse than the originally noted color. The same deepening of color was noted by investigators of this study during colony counting, potentially impacting characterization and enumeration of target organisms [86].

In the second objective of the study, concentrations of *E. coli* and coliform presumed to be ESBL resistant and concentrations of *E. coli* and coliforms presumed to be KPC resistant, were calculated and expressed as CFU/100 mL. Proportions of presumptive ESBL and KPC resistant bacteria relative to total *E. coli* and coliform concentrations were also calculated for each resistance type, per sample, per target organism (*E. coli* and other coliforms) and per collection event relative to total *E. coli* and coliforms enumerated in the same samples.

High concentrations of presumptive *E. coli* and other coliforms were found in all hospital sewage samples (N = 24) and presumptive ESBL positive and KPC positive *E. coli* and other coliforms were consistently found. The average concentrations of presumptive ESBL and KPC positive *E. coli* for all hospital sewage samples combined were 5.00×10^5 and 1.80×10^5 CFU / 100 mL, respectively. The average concentration of *E. coli* cultured on Bio-Rad agar during the same assays was 2.27×10^6 CFU / 100 mL. The average proportions of ESBL and KPC *E. coli* were 27.0% and 4.8%, respectively (Table 3.4). When hospital site-specific Wilcoxon-signed rank tests comparing the concentrations of ESBL and KPC producing *E. coli* were performed, no significant differences were found in Hospital Sites 1 -3 at a 95% CL, suggesting similar magnitudes of resistance in hospital sewage *E. coli* three of the four sites (Table 3.5). Kuskal Wallis tests comparing presumptive *E. coli* concentrations for all hospital sites on all three media also showed no significant differences between the hospital sites, indicating relative consistency (Table 3.20).

The average concentrations of presumptive ESBL and KPC positive non-*E. coli* coliforms in all hospital sewage samples combined (N=24) were 1.62×10^6 and 8.89×10^5 CFU / 100 mL, respectively. The average concentration of non-*E. coli* coliforms cultured on Bio-Rad agar during the same assays was 7.74×10^8 CFU / 100 mL. The average proportions of ESBL and KPC non-*E. coli* coliforms were 44.4% and 28.4%, respectively (Table 3.6). A Kruskal Wallis test comparing presumptive non-*E. coli* coliform concentrations for all hospital sites on all three media showed no significant differences between the hospital sites on Bio-Rad and KPC media, but not on CHROMagar ESBL (Table 3.20). This significant difference is derived from the variability of the mean concentrations of presumptive ESBL non-*E. coli* coliform in the four hospital samples. Each of these collection sites serve distinct wings of the hospital, thus conveying the waste and waste water of different types of patients, services, procedures, and employees. The observed variability in concentrations of target organisms may be due to several factors such as dissimilar antibiotics use and stewardship, severity and type of illnesses seen, length of stay at hospital, as well as the relative dilution of collected sewage[87–89]. These dynamics are beyond the scope of this study, but their determination would address a knowledge gap within the literature.

Hospital samples were collected near the points of human excretion, directly adjacent to each ward without any holding time or treatment infrastructure. One would expect to find highly resistant organisms of clinical significance in the hospital sewage, as it was likely to be excreted from patients with severe infections [70]. The high bacterial load and frequency of AMR in hospital wastewaters are comparable to several previous studies, including Prado et al. 2011 and Korzeniewksa and Harnisz 2013 who found bacterial loads of upwards of 7.1 X 10^{10} CFU / 100 mL in a Brazilian hospital sewage and 2.72 10^{7} CFU / 100 mL in Polish hospital sewage[87,90]. The UNC hospital sewage does not receive on-site treatment prior to introduction into municipal sewage systems and represents a large potential contribution to the possible further dissemination of AMR. This lack of treatment at hospitals is typical, exacerbating the dissemination potential and resulting health impact of AMR[49].

High concentrations of presumptive *E. coli* and other coliforms were found in all municipal sewage samples (N = 10) and presumptive ESBL positive and KPC positive *E. coli* and other coliforms were consistently found. Sewage samples were taken at the facility and represented a mix of several different sources with microbial interactions occurring both during transit and during initial removal of large solids and debris. The average concentrations of presumptive ESBL and KPC positive *E. coli* in municipal sewage were 2.70 x 10^{5} and 6.43 x 10^{4} CFU / 100 mL, respectively. The average concentration of presumptive *E. coli* cultured on Bio-Rad agar during the same assays was 2.58 x 10^{6} CFU / 100 mL. The average proportions of ESBL and KPC *E. coli* were 12.19% and 2.76%, respectively. The average concentrations of ESBL and KPC positive non-*E. coli* coliforms were 1.17 x 10^{6} and 2.37 x 10^{5} CFU / 100 mL, respectively. The average concentration of non-*E. coli* coliforms cultured on Bio-Rad agar during the same assays was 3.34 x 10^{7} CFU / 100 mL. The average proportions of ESBL and KPC positive other coliforms were 4.40% and 1.02%, respectively (Table 3.10).

At OWASA WWTP, the influent was exposed to primary, secondary and tertiary treatment by physical, biological and chemical processes, including chlorine disinfection. Such treatment creates pressures that could modify the characteristics and resistance profiles of the organisms still present after treatment. The average concentrations of presumptive ESBL and KPC positive *E. coli* in the secondary

effluent were 2.89 x 10^2 and 1.53 x 10^2 CFU / 100 mL, respectively. The average concentration of presumptive *E. coli* cultured on Bio-Rad agar during the same assays was 3.93 x 10^4 CFU / 100 mL. The average proportions of ESBL and KPC *E. coli* were 3.50% and 1.78%, respectively. The average concentrations of ESBL and KPC positive other coliforms were 1.11 x 10^3 and 1.10 x 10^3 CFU / 100 mL, respectively. The average concentration of non-*E. coli* coliforms cultured on Bio-Rad agar during the same assays was 9.58 x 10^4 CFU / 100 mL. The average proportions of ESBL and KPC positive other coliforms were 1.31% and 1.23%, respectively (Table 3.12).

A Mann-Whitney U test was performed to compare the proportions of presumptive ESBL and KPC positive target organisms in raw sewage and secondary effluent. Results indicated that there was not a significant difference in the proportions of ESBL and KPC positive *E. coli* and KPC other coliforms but a significant difference in the proportion of ESBL other coliforms between raw sewage and secondary treated effluent (Tables 3.10, 3.12 and 3.21). In this study, there was a higher proportion of presumptive ESBL other coliforms found in raw sewage, based on presumptive concentration and not on the proportion of confirmed bacterial isolates taken from samples. A 2006 Chilean study by Silva et al. derived the relative frequency of transferable multiple antibiotic resistance in confirmed coliform bacteria isolated from raw and treated sewage and found a higher overall frequency of resistance in the treated effluent and not the raw sewage[88]. In a study by Reinthaler et al. 2010, sewage from five different Austrian sewage plants were analyzed for ESBL *E. coli* to investigate the degree of contamination as well as the efficacy of different treatment methods and found that ESBL *E. coli* can still persist even after treatment [16]. In a 2013 study by Brechet et al., the raw sewage in a Parisian WWTP and sewage network was found to have higher concentrations of ESBL positive *E. coli* than were collected at the hospital. The authors suggest that this may be due to hospital water use, leading to overall higher dilutions in the samples and potential development and transferal of resistance during sustained containment at the WWTP [91].

Higher magnitude of confirmed antimicrobial resistance may be associated with the ephemeral nature of the hospital sewage in comparison to the longer-term residence times of community raw

sewage and secondary effluent samples[79]. Hospitals release a high concentration of antibiotics into municipal WWTP via hospital effluent. This creates selection pressure in these systems that sustain and foster AMR[92]. Presumptive ESBL positive *E. coli* and other coliform were found in 9 out of 10 secondary effluent samples. Likewise, presumptive KPC positive *E. coli* and other coliforms were found in 9 out of 10 secondary effluent samples. This is a concerning finding, as treated effluent is discharged into a water body that is hydrologically connected to an important drinking water source and recreational area.

The average concentrations of presumptive ESBL and KPC positive *E. coli* in Morgan Creek, upstream of WWTP were 2.48×10^1 and 9.13×10^1 CFU / 100 mL, respectively. The average concentration of presumptive *E. coli* cultured on Bio-Rad agar during the same assays was 4.02×10^2 CFU / 100 mL. The average proportions of ESBL and KPC *E. coli* were 3.18% and 3.19%, respectively (Table 3.14). The average concentrations of presumptive ESBL and KPC positive *E. coli* in Morgan Creek, downstream of WWTP were 6.03×10^1 and 3.17 CFU / 100 mL, respectively. The average concentration of presumptive *E. coli* cultured on Bio-Rad agar during the same assays was 1.29×10^3 CFU / 100 mL. The average proportions of ESBL and KPC *E. coli* were 11.55% and 1.89%, respectively (Table 3.16).

The average concentrations of ESBL and KPC positive other coliforms in Morgan Creek upstream of the WWTP were 1.77×10^2 and 3.89×10^2 CFU / 100 mL, respectively. The average concentration of presumptive non-*E. coli* coliforms cultured on Bio-Rad agar during the same assays was 1.52×10^4 CFU / 100 mL. The average proportions of ESBL and KPC positive other coliforms were 2.11% and 1.43%, respectively (Table 3.14). The average concentrations of ESBL and KPC positive other coliforms in Morgan Creek downstream of the WWTP were 3.70×10^2 and 1.42×10^2 CFU / 100 mL, respectively. The average concentration of presumptive non-*E. coli* coliforms cultured on Bio-Rad agar during the same assays was 3.12×10^4 CFU / 100 mL. The average proportions of ESBL and KPC positive other coliforms were 1.92% and 0.87%, respectively (Table 3.16).

When both the proportions and concentrations of KPC and ESBL positive *E. coli* and other coliforms were compared between the upstream and downstream site using a Mann-Whitney U test, no significant difference was found at a 95% CL (Tables 3.26 and 3.27). The relative consistency of AMR

loads and resistance characteristics of collected isolates from surface water samples impacted by WWTP effluent is also comparable to other studies that show there are other sources influencing the AMR load outside of effluent discharge. This was found by Blaak et al. in a 2014 Dutch study, Harnisz in a 2013 Polish study, Goñi-Urriza et al. in a 2000 Brazilian study all focused on the comparison of upstream and downstream resistance characteristics [45,78,93].

The third and fourth objectives of this study focused on the performance evaluation of CHROMagar ESBL and KPC agar medium for the detection and enumeration of ESBL and KPC positive *E. coli* and other coliforms in hospital sewage, raw sewage, treated sewage effluent and surface waters impacted by WWTPs. Overall, CHROMagar ESBL performed well in hospital sewage, raw sewage and secondary effluent samples. There was a high level of identity confirmation in these samples for presumptive coliforms, but not for *E. coli* via MALDI-TOF MS speciation. Many of the presumptive *E. coli* isolates were identified as "other coliforms" with exact identities found in Appendix 3. During VITEK 2 analysis, there was high confirmation of resistance to Cefpodoxime, a cephalosporin antimicrobial, in all sample for identified coliforms. In addition, many of the same isolates were also confirmed to have resistance to Imipenem, a carbapenem antimicrobial, and harbor multi-resistance.

Overall, CHROMagar KPC did not perform as well in environmental samples when compared to the confirmation rates from CHROMagar ESBL medium. There was a high level of identity confirmation in the evaluation of hospital sewage, raw sewage and secondary effluent samples for presumptive coliforms, but not for *E. coli* via MALDI-TOF MS speciation. Many of the presumptive *E. coli* isolates were identified as "other coliforms" with exact identities found in Appendix 4.

None of the surface water isolates were confirmed as coliforms, but were instead identified as other Gram-negative bacteria, many of which harbor intrinsic carbapenem resistance traits (Tables 3.51 - 3.55) [58]. During Modified Hodge testing, there was high confirmation of KPC presence in raw sewage and secondary effluent (Tables 3.42 – 3.50). Of the forty-four raw sewage confirmed non-*E. coli* coliform isolates that received both MALDI-TOF and Modified Hodge Testing, 82% were positive for KPC, 11% had "intermediate" levels of KPC, and 7% were negative (Table 3.45). Of the thirty secondary effluent

confirmed non-*E. coli* coliform isolates, 80% were positive for KPC, 10% had "intermediate" levels, and 10% were KPC negative (Table 3.49).

None of the twelve raw sewage or sixteen secondary effluent isolates of presumptive *E. coli* were confirmed via MALDI-TOF (Tables 3.44 and 3.48). These isolates were identified as other coliforms such and other Gram-negative bacteria such as *Serratia marcescens, Stenotrophomonas maltophilia,* and *Aeromonas caviae* (Appendix 4). All of the aforementioned bacteria are known to harbor resistance to carbapenems, are of clinical concern, and demonstrate clear to pink growth on CHROMagar Orientation [58,86,93–95].

Rapid identification of an infectious organism is invaluable for the administration of appropriate patient treatment, as well as useful for evaluating microbial source characteristics and transmission pathways. Before the introduction of chromogenic agar media in the 1990s, an array of phenotypic characteristics and simple biochemical tests were required to identify bacteria. Culture-based agar media have evolved considerably from nutrient, non-selective agar to the two substrate-specific chromogenic agars compared in this analysis, both designed to detect specific bacteria or groups of bacteria [3,96].

Modern chromogenic agar media contain specific substrates that impart differential, genus-specific (and sometimes species-specific) indicator properties that facilitate expedited identification of certain bacteria based on the color of their colonies[96]. The color comes from the hydrolysis product of the carbohydrate substrate-chromogen complexes that were generated by the reactions of genus- or species-specific enzymes. A requisite utility of all the culture agar media to be used in the proposed method is the capacity to discriminate common, clinically significant bacteria present in mixed cultures in environmental specimens after direct plating from representative waste waters and surface waters.

E. coli and other coliform bacteria are fitting indicator organisms, as they are presently used in many parts of the world for surveillance of fecal contamination as well as to monitor the efficacy of treatment and relative quality of waste and drinking waters. In particular, pathogenic strains of *E. coli* are known to be major contributors to community-acquired infections and *Klebsiella pneumonia* has been identified as having an important role as a cause of infections and illnesses in hospital settings. β-lactam antibiotics

are among the most important clinical antibiotics. Bacteria with resistance to Extended-spectrum β-lactams like cephalosporins and/or resistance to carbapenems can have significant medical consequences. Detection of ESBL and KPC production in bacteria indicates significant mechanisms of AMR, making detection of ESBL and KPC production in coliform bacteria an appropriate indicator system for evaluation of environmental AMR presence, concentrations and significance.

Antibiotic resistant bacteria have a series of phenotypic traits that increase their persistence and resistance. The base medium of CHROMagar Orientation supported the growth of interfering organisms that harbor like-resistance profiles. During the 13 assays, complex colonial characteristics were observed that both aided and hindered identification of the bacteria in question. Though similar in color, colonies varied in size, opacity, elevation, shape and consistency within the same plate. Pure colonies from surface water did not always exhibit the same properties as pure colonies from the other samples, making it very important to learn, acknowledge and include the sample source in culture examination and judgement.

A 1996 study of CHROMagar Orientation found that mucoid *E. coli* and *K. pneumoniae* strains often masked underlying growth of different organisms and that the medium may not be suitable for direct isolation[86]. Mucoid colonies were often seen in hospital sewage, raw sewage and secondary effluent and were initially very difficult to isolate and characterize, as confluent mixtures of non-mucoid colonies often had the same appearance. Colonies of presumptive target organisms cultured from surface water samples on the antibiotic media were often small and dry in initial appearance. Although colony isolates were frozen for later confirmation and characterization, isolates from surface water samples had poor revival rates, often requiring multiple subcultures before validation steps commenced.

One potential reason for the high number of false positives as well as low isolate revival for *E. coli* is that the ESBL and KPC CHROMagar media rely on β-D-Glucuronidase (GLUC) activity to differentiate *E. coli.* All the CHROMagar products used in the study as well as Bio-Rad Rapid'*E. coli 2* agar medium use β-D-Galactosidase (GAL) specific chromogenic substrates and β-D-Glucuronidase (GLUC) specific chromogenic substrates to differentiate coliforms and *E. coli*, respectively[86,97]. When coliforms cleave the GAL substrate, a green precipitate is left behind as the enzyme hydrolysis product, allowing for positive

confirmation of coliforms. When *E. coli* cleave the GLUC substrate, a pink precipitate is left behind as the enzyme hydrolysis product, allowing for positive confirmation of *E. coli* [98]. CHROMagar Orientation has an additional proprietary blend of other chromogenic substrates used for the detection of both Gram-positive and -negative organisms [86]. A full listing can be found in Table 2.5. *E. coli* specific survival and disinfection studies have shown that loss in cultivability does not necessarily result in the loss of GLUC enzyme activity, resulting in false positives for uncultivable bacteria [95,99]. Other false positives may be related to the production of GLUC and GAL by many organisms other than *E. coli* and coliforms, respectively. GLUC can be produced by *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp. and *Streptococcus* spp. all of which were identified in presumptive *E. coli* isolate samples, especially when isolated on the CHROMagar KPC medium[95].

Limitations

Funding and Confirmation of Bacteria Isolates

Funding was the major limiting factor that hindered timely confirmation and characterization of presumptive colonies, possibly reducing the accuracy, precision and reliability of the subjective presumptive bacteria counts and concentration calculations. Confirmatory antimicrobial susceptibility testing and speciation should have been performed prior to freezing the initial isolates or much earlier in the project to better evaluate the performance of the media, the experimental protocol and the proficiency of the analysts involved. The antimicrobial resistance profile of an organism can change with stress, prolonged storage at sub-optimal conditions and repeated serial culturing [100]. Repeated freeze/thaw and exposure to antibiotics during revival for further testing may damage bacterial cells and possibly increase or change initial susceptibility of the test isolate [100]. Such changes from repeated isolate handling could bias the performance evaluation of the media using conventional antimicrobial susceptibility techniques.

Analyst Training and Proficiency

Successful performance of the proposed culture methods requires knowledge of culture-based, clinical-style microbial techniques that the analyst team initially lacked, as well as proper performance

and consistent incorporation of quality control measures. Prior to training at UNAN in the summer of 2015, the team responsible for the evaluation of this method did not have the preferred level of training, skill and proficiency required for the reliable detection, enumeration and isolation of wild-strain resistant target organisms, many of which displayed an array of highly variable phenotypic characteristics.

For instance, it may have been concluded that two blue colonies had the same presumptive identities. However, with in-service training and experience in getting results from speciation, one could discern that one apparent "colony" was a presumptive turquoise *Enterococcus* colony merging over the smaller pink *E. coli* colony, and the other apparent "colony" was a metallic blue *Klebsiella*-type colony covered in cream, pin-point *Pseudomonas* colonies. These are prototypical examples of several issues that can lead to poor bacteria isolation success and subsequent contamination of frozen isolates. It is also illustrative of the training and experience needed to successfully utilize and evaluate this, or a similar, culture-based method.

Sample Comparisons and Size

The sampling strategy lacked temporal consistency between the hospital and other sample types due to delays in the participation of the UNC hospital system. This delayed inclusion of hospital samples into the regime by five months hindered the simultaneous comparison of all sample types. Jordan Lake was also sampled less due to limited sample collection and analysis capacity. Power is based on the smallest sample size and our sampling limitations reduced the power of both the paired Wilcoxon Signed Rank and Kruskal Wallis analysis of some of the samples. The sample size for the hospital samples, while on the cusp of being too small, allowed for the Kruskal Wallis inter-sample comparison. A Kruskal Wallis analysis of Morgan Creek (upstream and downstream) with Jordan Lake was not done due to unequal sample sizes that would affect the homogeneity of variance assumption. Though a moderate departure from sample size is possible, it is not as rigorous, as the results are considerably less robust.

Reliance on GAL and GLUC for differentiation of Coliforms and E. coli

Beta-D-Galactosidase (GAL) has been found in numerous gram-negative bacteria strains belonging to *Enterobacteriaceae and Pseudomonadaceae* as well as in several gram-positive bacteria,

yeasts, protozoa, and fungi [95,101]. Beta-D-Glucuronidase (GLUC) is produced by most *E. coli* strains and also by other members of *Enterobacteriaceae*, including some *Citrobacter* and *Edwardsiella* as well as by *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp. and *Streptococcus* spp. [3,95].

All the CHROMagar media and the Bio-Rad Rapid'*E. coli 2* agar medium use β-D-Galactosidase (GAL) specific chromogenic substrates and β-D-Glucuronidase (GLUC) specific chromogenic substrates to differentiate and positively identify coliforms and *E. coli* colonies, respectively. Sole dependence on the green and pink colored precipitate as evidence for positive identification of coliforms and *E. coli* without use of other confirmatory methods may be in error, as production of GAL and GLUC can occur in other, non-target organisms, thereby creating false positives during the detection and enumeration of coliforms and *E. coli* in environmental matrices.

Recommendations

The expressed limitations of this study highlight the need for more funding, better test media and engagement of clinical and veterinary microbiologists for better evaluation. Though the proposed method is generally promising, culture-based methods can be onerous, time-consuming and prone to bias when compared to other available, more sensitive and rapid detection methods. PCR is a highly sensitive and specific method for the detection of AMR, but remains unavailable for use in many laboratories [19]. A tiered approach that utilizes both molecular and phenotypic methods is needed. Using similar methods to the ones evaluated in this study would address capacity limitations and thereby be inclusive of a broad network of stakeholders required to fully implement the proposed global surveillance system.

It is pragmatic and prudent to adhere in part to highly standardized, culture-based clinical methods for assessing antibiotic resistance, as their use would support, rather than hinder, the broad adoption and implementation of future surveillance efforts. Accordingly, building from culture-based methods already used in clinical laboratories around the world is a pragmatic and prudent strategy towards globally harmonized surveillance. More work should be done toward the development or selection of appropriate and effective culture-based media. If a harmonized, culture-based methodology

for the surveillance of AMR in representative waste and environmental samples is to be developed, serious consideration must be given to its availability, expense, infrastructure requirements, relative time burden, and the choice, availability and effective use of its culture media. Without the incorporation of recommendations outlined below, a globally coordinated AMR surveillance system will possibly never come to fruition.

Initial and Iterative Performance Evaluation

Determination of resistance profiles and bacterial identities of presumptive isolates should occur as soon as possible upon initial bacteria detection, enumeration and isolation from the samples. The resistance profile of an organism can change with stress or poor storage conditions. Repeated freeze / thaw and exposure to antibiotics during revival for future testing may damage bacterial cells, increasing or causing changes in the initial susceptibility of the test isolate, biasing the performance evaluation of the media using conventional antimicrobial susceptibility techniques[102]. Antimicrobial susceptibility testing in clinical laboratories is routine, expected and standardized. Future evaluation of this method should be done in clinical laboratories where the assays are routine, highly structured and more representative of the conditions in which the future AMR surveillance method would be adopted and encouraged.

Bacteria Colony Color and Morphology Referencing Catalog

A bacteria colony color and morphology referencing catalog should be developed at the beginning of the project and updated iteratively with continued confirmation of presumptive identities. Though perhaps challenging to the capacity of many labs, including this one, a series of control bacteria isolates or strains with similar resistance traits to the target organisms should be performed on the medium in question, with referencing of colony appearance using a Pantone international printing color chart. Another option would be to take high resolution photographs of the colonies on the resulting test plates for later comparison and then import them to a digital device (e.g. mobile phone or tablet) and / or obtain high quality, printed copies). This use of reference and positive control bacteria strains should also be done proactively with environmental bacterial isolates that will be immediately characterized. The colony photos would be linked to the bacterial identity and sample type and used to guide interpretation in future assays. If different morphological characteristics are attributed to the same bacteria species

after rigorous characterization, a photo and description would be added, creating an interpretation catalog for future use by the researchers as well as others.

Cost and Availability of Indicator System

Further evaluation of the CHROMagar agar media must be done before definitive statements can be made regarding their suitability for use in the discussed monitoring method of a surveillance system. CHROMagar Orientation, KPC and ESBL are very expensive and may create serious cost barriers for many laboratories in low-resource settings. CHROMagar Orientation is the base media for CHROMagar ESBL and KPC. Purchasing a five liter supply of both CHROMagar ESBL and KPC agar media with requisite antibiotic supplements costs approximately $800 USD, with CHROMagar ESBL agar costing $385 and CHROMagar KPC agar costing $443, respectively. A five liter supply of BioRad Rapid'*E. coli 2* agar costs $412 USD. This does not include the cost of consumables or other evaluation and confirmation analyses. Other clinical candidate agar media are being identified for incorporation into this ongoing study. However, the fact remains that antibiotic supplements are very expensive and require refrigeration. Validating the proposed protocol requires three step-wise uses of the antibiotic containing medium: one for initial spread plating or membrane filtration and two additional purification streaks on agar media plates. This can become very costly and possibly impede consistent culture-based monitoring for surveillance.

Many clinical laboratories, especially in low- and middle-income countries, are capable of overcoming the lack of availability and / or expense of bacteriologic culture media by preparing comparable media from "scratch" from listed, constituent ingredients. However, the antibiotics and chromogenic substrates in CHROMagar media are proprietary and unlisted. Due to the cost and proprietary nature of many antibiotic substrates, iterative plating, post-spreading or filtration, may not be economically feasible. Thus, it is recommended to find alternative agar media for the purification steps that is capable of differentiating target resistant species. For instance, MacConkey agar is the most widely used clinical medium in clinical laboratories for the differentiation and isolation of Gram-negative bacteria and has been shown to perform well in disk diffusion tests for the phenotypic evaluation of KPC

production[65]. It can be supplemented with known ESBL and KPC antimicrobials to select for and purify such bacteria isolates at lower initial cost. However, it does not allow direct and specific visual distinction of *E. coli* from other coliforms, unless a chromogenic or fluorogenic Beta-D-glucuronide substrate is incorporated into the medium. Furthermore, the use of such culture media made from "scratch", including the preparation and storage of the antimicrobials to be used with them, requires rigorous and regular quality control and quality assurance activities, which also adds cost, time and effort.

Animal-free products

All of the agar media used in the project have animal-derived ingredients. Consequently, a global-scale harmonized methodology would exclude use in countries or cultures that practice strict vegetarianism or do not use pork-based or beef-based products, e.g. certain sects of Hinduism, Buddhism, Judaism, and Islam. To create an inclusive methodology, animal-free products should be identified and incorporated into the methodology or validated against the performance of animal-containing products.

Spread Plating vs. Membrane Filtration

Inclusion of traditional approaches for isolation and quantification of microbial indicators will continue to be relevant. Spread plating on standard size plates of agar media supplemented with antibiotics at MIC is more appropriate for enumeration of AMR in wastewater samples that are expected to have higher bacterial concentrations. In membrane filtration on 60 mm diameter culture plates overgrowth of non-target organisms and biofilms was routinely observed during this project. Side experiments were done to evaluate the efficacy of spread plating and this method was found to unequivocally maximize the probability of obtaining distinct, isolated colonies when compared to membrane filtration. However, if surface water or other samples that are expected to be less contaminated are analyzed, the membrane filtration technique is still preferred to spread plates, as the latter requires analysis of larger volumes of water. In addition, more work should be done to develop and evaluate a liquid cultivation and quantitative method that utilizes multiple tube techniques and most probable number calculations. This would better contend with more turbid samples as well as allow for a more flexible and broad range of sample volumes.

Partners, Personnel, and Planning

A One Health approach would involve multi-sector involvement in AMR monitoring for integrated surveillance, such as project partnership between clinical, veterinary-agricultural and environmental microbiology labs and researchers. Planning is requisite due to the infectious nature of domestic and hospital sewage samples, and differences in analytical techniques, and materials and methods. These differences should be identified and overcome early so as to identify harmonized methods in planning, training and implementation. A standard operating procedure must be developed prior to project initiation for sample collection, transport, storage, processing, analysis and enumeration and confirmation. All personnel involved must be fully trained prior to project initiation, both in the lab and in the field.

The determination of sampling sites and collection strategies should be based on accessibility, consistency and institutional capacity. Composite sampling, or collection of multiple, discrete samples taken at a regular interval over a pre-determined time, can potentially provide better, more representative sampling of a waterbody or waste treatment facility when compared to grab samples. Composite or repeated samples better represent a system through repeated sample collection over time rather than at a single instant. However, composite sampling is much more capacity- and time-intensive. Grab samples, though less representative, are a less-intensive alternative to characterize water quality at the point and specific time of collection. It is important to consider that sampling waste and surface water frequently requires the direct involvement of, and coordination with, external parties, e.g. treatment plant or hospital facilities personnel for waste water samples and landowners for surface water samples. Establishing and finalizing the terms of external involvement is essential but may require more time than expected. It is also important to address potential administrative-related delays in project planning and implementation.

Future Work

There are several procedures that could be changed in order to create future improvements in the culture-based detection and enumeration of ESBL and KPC *E. coli* and coliforms in samples of wastewater and environmental surface water as an ongoing effort. More work is being done to create,

further evaluate and attempt to validate an accessible, final methodology for direct, culture based detection and enumeration of ESBL and KPC *E. coli* and coliforms in wastewater and environmental waste samples. A revised protocol is in review and will be made available for open distribution to present and future partners and other interested parties and stakeholders. Future work includes application of the revised methodology to matrices covered in One Health strategies, such as agricultural waste, manure, aquaponics and animal carcasses.

Summary and Conclusion

Modern antibiotics have changed the world by treating infectious diseases that were once fatal, saving millions of lives. However, in the seventy years since their introduction, antibiotics have been used excessively and indiscriminately in medical, agricultural, veterinary and domestic settings. This irresponsible and imprudent use has introduced antibiotic waste into the environment, creating massive reservoirs of ARB presence and complex systems of selective pressure that foster, sustain and spread AMR and ARG from bacteria to bacteria, person to person or animal, animal to animal or person, setting to setting and country to country. With the global spread of AMR, the world is facing a post-antibiotic era where even the simplest infections may become fatal[18]. Little is known about the actual magnitude of AMR because the resistome is vast and multi-faceted with pathogens and autochthonous bacteria harboring, transferring and acquiring AMR and ARG to the most sophisticated chemotherapies at rates rapidly exceeding pharmacological development of new antimicrobial agents.

Continuous identification, evaluation and monitoring of AMR hotspots are incredibly important to combatting and preventing the spread of AMR. Hospital settings are often considered one of the most important reservoirs for the spread of AMR, and mitigation strategies such as antibiotic stewardship have been implemented. However, wastewater treatment systems are potentially as important as hospitals, and the efficacy of such treatment is of pressing concern. When fecal wastes from patients are conveyed to municipal wastewater treatment plants, the AMR pathogens contained within the wastes follow. Evaluating the magnitude of AMR in influent is a potential proxy for evaluating the magnitude of AMR in the community, analogous to obtaining a snapshot community-wide fecal sample. Evaluating the

presence of AMR in treated effluent and biosolids can also be used to evaluate the efficacy of treatment as well as the risk of emission of AMR into the environment. Focus on WWTP hotspots is not always appropriate, as many areas do not have safe wastewater containment, management or treatment. Surface and other environmental waters should also undergo surveillance, as it is estimated that 90% of wastewater is discharged untreated into the environment[68]. This is especially important if land application of human and animal waste is practiced. These unmitigated releases work to further increase and spread AMR and ARG, exacerbating the risk of exposure and potential burden of infectious disease.

There are presently no internationally harmonized or enforced strategies, guidelines and standards in place that address AMR prevention and reduction in medical, domestic, agricultural, veterinary and food-production settings. Consequently, there are no harmonized strategies for requisite evaluation, monitoring and control of AMR, creating multiple barriers to the institutionalization and maintenance of aforementioned policies, guidelines and standards. WHO and others have called for coordinated global action to combat AMR, emphasizing surveillance methods that are accessible and practicable across the world [1,12,18]. Deterrents to the development of this surveillance method include disparate capacities of labs, the lack of standardized antimicrobial detection, quantification and susceptibility testing methods and differences regarding the use of target indicator organisms and target antimicrobial resistance traits to detect and possibly quantify.

The primary goal of this Masters research was to advance a proof of concept indicator system proposed by the World Health Organization via performance evaluation of a prototype method for the direct, one-step, culture-based quantitative detection of ARB in representative waste waters and environmental samples of interest. The proposed method can be briefly characterized as a culture-based indicator system for the direct detection and quantification of Extended-spectrum-β-lactamase (ESBL) and carbapenemase (KPC) producing *Enterobacteriaceae* of the coliform group in exposure-relevant environmental matrices such as hospital and domestic sewage, treat sewage effluent, surface waters, and agricultural waste. The work was divided into four research objectives (Tables 1.1 – 1.4) that were executed in eighteen months over five different project phases (Figure 2.6).

The first objective was to assess the performance of CHROMagar Orientation – a chromogenic clinical diagnostic medium used for the isolation and differentiation of urinary tract pathogens – as the primary agar medium in the prototype method. CHROMagar Orientation medium was chosen above several other comparable media due to the availability of two corresponding product lines of bacteriologic culture media: CHROMagar ESBL for the detection of Gram-negative bacteria producing ESBL and CHROMagar KPC developed for the detection of Gram-negative bacteria with reduced susceptibility to most carbapenem agents. Both media were designed for use in the evaluation of urine and stool samples and are composed of CHROMagar Orientation as the base medium supplemented with a proprietary mixture of either extended-spectrum-β-lactam or carbapenem antibiotics.

The performance assessment involved repeated, parallel assays of representative environmental and waste water samples using procedures of EPA Method 1604 for membrane filtration followed by culturing on CHROMagar Orientation and Bio-Rad Rapid'*E. coli* 2. Bio-Rad Rapid'*E. coli* 2 is a chromogenic, culture-based medium used for the detection and enumeration of *E. coli* and other coliform bacteria in food and waste waters and was used as the "gold standard" for performance. The resultant concentrations of presumptive *E. coli* and other coliforms from both media were paired by date, sample site and organism and compared using a Wilcoxon signed-rank tests, with a significance $\alpha = 0.05$. Results suggest that:

Objective 1 results:

- The performance of the clinical medium was comparable to the gold standard for detection of *E. coli*, non-*E. coli* coliforms, and combined coliforms in hospital sewage and treated sewage effluent matrices (Tables 3.1 – 3.3), fulfilling the requirements for the proposed method associated with discrimination of target bacteria in mixed culture of environmental matrices.

- The performance of the clinical medium was comparable to the gold standard for detection of non-*E. coli* coliforms and combined coliforms in raw sewage and surface water. (Tables 3.2 – 3.3)

- The performance of CHROMagar Orientation in raw sewage and surface water matrices for the detection of *E. coli* did not fulfill the needed requirements for the proposed method associated with discrimination of target bacteria in mixed culture of environmental matrices. (Table 3.1)

The second objective was to determine the presence, concentration, and relative proportion of presumptive ESBL producing *E. coli* and other coliforms as well as presumptive carbapenemase producing *E. coli* and other coliforms in the same representative waste waters and environmental surface waters. During the aforementioned assays, the same samples were also plated on the CHROMagar ESBL and CHROMagar KPC media. The resulting concentration data was used to calculate a relative proportion of presumptive ESBL producing *E. coli* and coliforms as well as presumptive carbapenemase producing *E. coli* and other coliforms. This was done by pairing ESBL or KPC concentrations with Bio-Rad data from the same date, sample site and organism. Proportions for each resistance profile were then calculated using either the ESBL or KPC concentrations as the numerator and the Bio-Rad concentrations as the denominator.

Objective 2 results:

- ESBL and KPC *E. coli* and other coliforms are present in hospital sewage, domestic sewage, treated sewage effluent, and surface waters up and downstream from the effluent discharge point.
- ESBL and KPC producing *E. coli* and other coliforms can be consistently detected and confirmed at high concentrations in hospital sewage and raw sewage
- Lower, but still detectable concentrations of presumptive ESBL and KPC producing bacteria can be found in secondary effluent and surface water samples
- Secondary treatment of the sewage does not completely eliminate ESBL and KPC producing bacteria.

The third objective focused on validation of the CHROMagar ESBL agar medium via analysis and confirmation of frozen isolates of presumptive ESBL *E. coli* and non-*E. coli* coliforms collected during the aforementioned assays. Isolates were revived and subjected to VITEK 2 automated antimicrobial

susceptibility analysis in order to assess and confirm reduced susceptibility to extended-spectrum-β-lactam antibiotics. MALDI-TOF MS was then performed to speciate the same isolates for further confirmation of identity as presumptive indicator *E. coli* or non-*E. coli* coliforms.

The fourth objective centered on validation of the CHROMagar KPC agar medium via analysis and confirmation of frozen isolates of presumptive KPC producing *E. coli* and non-*E. coli* coliforms. Isolates were revived and subjected to Modified Hodge Testing for antimicrobial susceptibility analysis and to phenotypically evaluate resistance profiles, thereby confirming KPC production. MALDI-TOF MS was also used for speciation and identity confirmation.

Objective 3 and 4 results:

- The CHROMagar ESBL agar medium is capable of correctly detecting microorganisms with resistance to extended-spectrum-β-lactam antibiotics in all sampled environmental matrices (Tables 3.28 – 3.36)
- For all sample matrices, several organisms isolated on CHROMagar ESBL medium demonstrated multiple resistance to both extended-spectrum-β-lactam and carbapenem antibiotics (Tables 3.28 – 3.36).
- CHROMagar KPC medium did not perform as well in the sampled environmental matrices for the detection of indicator organisms with reduced susceptibility to carbapenem. There was high confirmation of KPC production in raw sewage and secondary effluent, but, given study and methodological limitations, more analysis should be performed (Tables 3.37 – 3.55).
- The performance of both CHROMagar ESBL and KPC media in environmental matrices for the correct detection of *E. coli* is unreliable due in part to the diversity and complexity of autochthonous organisms and reliance on Beta-D-Galactosidase and Beta-D-Glucuronidase for visual target colony differentiation and positive identification (Tables 3.28 – 3.55).
- Both media also performed poorly in surface water sites, however, a complex resistome may exist in the autochthonous bacteria analyzed. Many harbored intrinsic resistance at MICs breakpoints, but were not species considered of clinical concern within the CLSI database. This

may merit future evaluation and inclusion, as holistic surveillance of hotspots will require the analysis of this potential reservoir. (Tables 3.51 – 3.55 and APPENDIX 4).

- The use of a chromogenic substrate standard clinical agar medium for the direct detection and enumeration of *E. coli* and coliforms as fecal indicator bacteria in representative waste waters and surface waters is promising. However, given the limitations of the agar base CHROMagar Orientation, other candidate clinical media should be identified and evaluated for performance and suitability in the proposed method.

In conclusion, the development of new antibiotic chemotherapies has lagged over the past decade, suggesting a tipping point in pharmaceutical innovation. If this is indeed true, we must look to other approaches for mitigation of AMR development and spread. If one critically evaluates the advent of modern public health, it is the development and employment of antimicrobials in concert with improvements in the quality of and access to water, sanitation, and hygiene as leading achievements and accomplishments. This study demonstrates that present waste management methods are not sufficient for the containment and treatment of AMR. The elevated concentrations of highly AMR bacteria in hospital and municipal sewage as well as treated effluent indicates the widespread presence in the population and their possible spread to others from exposure via environmental, food and person-to-person transmission routes. More must be done to inform and support efforts to control and prevent the spread and release of antimicrobial agents, ARB, and ARG in human and animal waste into the environment. Coordination of surveillance efforts with documentation of antibiotic consumption provides a baseline for assessment efforts for future AMR mitigation strategies [103]

The global spread of ARB merits evaluation across other geographic regions in US and abroad using parallel, matching and harmonized methods to identify ARB threats and detect outbreaks. These media and methods have promise as a candidate indicator system to detect and quantify ARB of health concern in environmental media as a monitoring system to support environmental surveillance, thereby generating supporting evidence to inform and help shape future global action plans

The work performed during this study has implications for the development of future protocols for the analysis of AMR in the environment. To date, there are no published studies that evaluate the performance of CHROMagar ESBL agar and CHROMagar KPC agar for direct detection of presumptive ESBL and KPC positive *E. coli* and other coliforms in hospital sewage, municipal sewage, secondary effluent and surface waters by membrane filtration. There are also no published studies comparing the performance of the based agar medium, CHROMagar Orientation, to Bio-Rad Rapid'*E. coli 2* agar as the "gold standard" agar medium for direct detection and enumeration of *E. coli* and coliforms in environmental matrices by membrane filtration. This study provides significant information that supports the development and improvement of future prototype methods as well as evidence to inform future recommendations on candidate methods for direct, culture-based environmental monitoring using a harmonized and ongoing surveillance system for specified AMR traits of *E. coli* and other coliforms in the environmental samples that are the same as or consistent with the methods used in clinical and agricultural/veterinary settings and samples.

APPENDIX 1 MEDIA PREPARATION

Bacteriologic Agar Media

All agar media will be prepared according to manufacturer's instructions and plates were stored for no longer than 1 month at 4°C. CHROMagar ESBL and CHROMagar KPC antibiotic additives will be stored at 4°C and prepared according to manufacturer's instructions.

Bio Rad Rapid' E. coli 2 agar medium – A 37g quantity of powdered media per 1 liter of deionized water was mixed in a container. The agar medium was dissolved by bringing to a boil on a magnetic hot plate at a moderate temperature and the use of a stir bar for frequent agitation. The molten medium was then autoclaved at 121°C for 15 minutes. After sterilization, agar medium was tempered in a 55°C water bath for approximately 30 minutes and then poured into the desired plate size.

CHROMagar™ Orientation Medium – A 33 g quantity powder medium base in 1 liter of deionized water was mixed in container. The agar medium was dissolved bringing to a boil on a magnetic hot plate at a moderate temperature with a stir bar for frequent agitation. The molten medium was then autoclaved at 121°C for 15 minutes. After sterilization, the agar medium was tempered in a 55°C water bath for approximately 30 minutes and then poured into desired plate size.

CHROMagar™ ESBL - ESBL antibiotic supplement was prepared by weighing 570 mg ESBL supplement to 10 mL of cooled, autoclaved deionized water. Agar medium was prepared by adding reconstituted supplement solution to cooled, molten CHROMAgar Orientation medium at a 1% volume ratio (i.e. 10 mLs antibiotic supplement solution to 1 liter of agar). Supplement was not stored and used the same day it was prepared. Plates were poured as for the other agar media.

CHROMagar™ KPC – KPC antibiotic supplement was prepared by weighing 400mg KPC supplement to 10 mL of cooled, autoclaved deionized water. Agar medium was prepared by adding reconstituted supplement solution to cooled, molten CHROMAgar Orientation medium at a 1% volume

ratio (i.e. 10 mLs antibiotic supplement solution to 1 liter of agar) and plates were poured as for other media. Supplement was not stored and used the same day it was prepared.

Tryptic Soy Agar (Difco™) – A 40g quantity of powder medium per 1 liter of deionized water was mixed in container. To dissolve it the agar medium was then brought to a boil on a magnetic hot plate at a moderate temperature with a stir bar for frequent agitation. The medium was then autoclaved at 121°C for 15 minutes. After sterilization, the agar medium was tempered in a 55°C water bath for approximately 30 minutes and then poured into the desired plate size.

Mueller Hinton Agar (Remel™) – A 38g gram quantity of medium powder per 1 liter of deionized water was mixed in container. To dissolve it, the agar medium was then brought to a boil on a magnetic hot plate at a moderate temperature with a stir bar for frequent agitation. The medium was then autoclaved at 121°C for 15 minutes. After sterilization, agar medium was tempered in a 55°C water bath for approximately 30 minutes and then poured at 25 mL per 100 mm x 15 mm plate after the pH of agar medium was evaluated to ensure it was between 7.2 and 7.4 at room temperature.

MacConkey (Remel™) – A 50g quantity of powder medium per 1 liter of deionized water was mixed in container. To dissolve it, the agar medium was then brought to a boil on a magnetic hot plate at a moderate temperature with a stir bar for frequent agitation. The agar medium was then autoclaved at 121°C for 15 minutes. After sterilization, the agar medium was tempered in a 55°C water bath for approximately 30 minutes and then poured into the desired plate size.

Stock Solutions and Other Media

Phosphate-buffered solution [81] To make phosphate buffer stock, dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in 500 mL reagent grade water and dilute to 1L. To make phosphate buffer, 1.25 mL of concentrated stock phosphate buffer solution and 5.0mL $MgCl_2$ solution (81.1g $MgCl_2 \cdot H_2O$/L reagent grade water) was added to 1 L reagent grade water and then autoclaved for 15 mi at 121°C and stored in 4°C cold room until use, for no more than 3 weeks.

Buffered Peptone Water (BPW) [81] – Quantities of 10.0g peptone, 5.0 g NaCl, 3.5 g Na_2HPO_4, and 1.5 g KH_2PO_4 were dissolved in in 1 L reagent grade water. pH was adjusted to 7.2 + 0.5 with 1N NaOH, and then the BPW was autoclaved for 15 mi at 121**°**C and stored in 4°C cold room until use, for no more than 3 weeks.

1X Trypticase Soy Broth *(TSB)*- A 30 g quantity of powder medium was dissolved in 1 liter deionized water, mixed thoroughly, and then heated on a hotplate until completely dissolved. TSA was then autoclaved at 121**°**C for 15 minutes and stored in 4°C cold room until use, for no more than 3 weeks.

40% Glycerol Solution – A 4 part glycerol to 6 part deionized water solution was prepared, by volume, and autoclaved at 121**°**C for 15 minutes. The solution was stored in 4°C cold room until use, for no more than 3 weeks.

APPENDIX 2 CONCENTRATION AND PROPORTIONS

Table 1.A - Hospital 1 (GI MICU) *E. coli* Concentration (CFU / 100mL) by Media and Date
Table 1.B - Hospital 1 (GI, MICU) Non- *E. coli* coliforms Concentration (CFU / 100mL) by Media and Date
Table 1.C - Hospital 1 (GI, MICU) Total coliform Concentration (CFU / 100mL) by Media and Date

Table 2.A – Hospital 2 (Burn, Lab, ICU) *E. coli* Concentration (CFU / 100mL) by Media and Date
Table 2.B – Hospital 2 (Burn, Lab, ICU) Non- *E. coli* coliforms Concentration (CFU / 100mL) by Media Date
Table 2.C – Hospital 2 (Burn, Lab, ICU) Total coliform Concentration (CFU / 100mL) by Media and Date

Table 3.A – Hospital 3 (Heart, ICU) *E. coli* Concentration (CFU / 100mL) by Media and Date
Table 3.B – Hospital 3 (Heart, ICU) Non- *E. coli* coliforms Concentration (CFU / 100mL) by Media and Date
Table 3.C – Hospital 3 (Heart, ICU) Total coliform Concentration (CFU / 100mL) by Media and Date

Table 4.A – Hospital 4 (ER, Neuro) *E. coli* Concentration (CFU / 100mL) by Media and Date
Table 4.B – Hospital 4 (ER, Neuro) Non- *E. coli* coliforms Concentration (CFU / 100mL) by Media and Date
Table 4.C – Hospital 4 (ER, Neuro) Total coliform Concentration (CFU / 100mL) by Media and Date

Table 5.A – Raw Sewage *E. coli* Concentration
Table 5.B – Raw Sewage Non- *E. coli* coliforms Concentration
Table 5.C – Raw Sewage Total coliform Concentration

Table 6.A – Secondary Effluent *E. coli* Concentration
Table 6.B – Secondary Effluent Non- *E. coli* coliforms Concentration
Table 6.C – Secondary Effluent Total coliform Concentration

Table 7.A – Morgan Creek Upstream *E. coli* Concentration
Table 7.B – Morgan Creek Upstream Non- *E. coli* coliforms Concentration
Table 7.C – Morgan Creek Upstream Total coliform Concentration

Table 8.A – Morgan Creek Downstream *E. coli* Concentration
Table 8.B – Morgan Creek Downstream Non- *E. coli* coliforms Concentration
Table 8.C – Morgan Creek Downstream Total coliform Concentration

Table 9.A – Jordan Lake *E. coli* Concentration (CFU / 100mL) by Media and Date
Table 9.B – Jordan Lake Non- *E. coli* coliforms Concentration (CFU / 100mL) by Media and Date
Table 9.C – Jordan Lake Total coliform Concentration (CFU / 100mL) by Media and Date

Hospital 1 Sewage (Gastrointestinal and Medical Intensive Care Unit):

Table 1.A – Hospital 1 (GI and MICU) *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid '*E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/11/2015	9	No BR	3.33E+06	5.67E+04	9.58E+04	1.70%	2.88%
5/18/2015	10	1.00E+06	1.43E+06	2.00E+04	2.50E+04	1.40%	1.74%
5/26/2015	11	2.15E+05	1.72E+06	1.35E+05	1.55E+04	7.83%	0.90%
6/15/2015	12	6.48E+06	3.30E+07	6.00E+06	2.23E+06	18.18%	6.74%
7/1/2015	13	1.40E+06	1.40E+06	8.00E+04	4.00E+03	5.71%	0.29%
Average		2.27E+06	8.18E+06	1.26E+06	4.73E+05	6.97%	2.51%
Standard Error		7.54E+02	1.28E+03	5.02E+02	3.08E+02	3.06%	1.14%
95% Confidence ±		1.48E+03	2.51E+03	9.83E+02	6.03E+02	5.99%	2.24%

Table 1.B – Hospital 1 (GI and MICU) Non-*E. coli* coliforms Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/6/2015	8	1.44E+09	4.48E+08	1.66E+07	1.10E+07	3.70%	2.46%
5/11/2015	9	No BR	3.33E+06	2.91E+06	2.52E+06	87.27%	75.48%
5/18/2015	10	3.50E+06	2.33E+06	1.32E+06	1.75E+06	56.71%	75.14%
5/26/2015	11	3.70E+06	2.54E+06	1.16E+06	8.75E+02	45.70%	0.03%
6/15/2015	12	6.79E+07	8.02E+07	9.93E+06	2.65E+06	12.38%	3.31%
7/1/2015	13	3.00E+05	3.40E+06	3.90E+05	5.20E+04	11.47%	1.53%
Average		3.04E+08	8.99E+07	5.38E+06	3.00E+06	36.21%	26.33%
Standard Error		7.80E+03	3.87E+03	9.47E+02	7.07E+02	13.36%	15.50%
95% Confidence ±		1.53E+04	7.59E+03	1.86E+03	1.38E+03	26.18%	30.38%

Table 1.C – Hospital 1 (GI and MICU) Total coliform Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/11/2015	9	No BR	6.67E+06	2.97E+06	2.61E+06	44.48%	39.18%
5/18/2015	10	4.50E+06	3.77E+06	1.34E+06	1.78E+06	35.66%	47.21%
5/26/2015	11	3.91E+06	4.27E+06	1.30E+06	1.64E+04	30.41%	0.38%
6/15/2015	12	7.44E+07	1.13E+08	1.59E+07	4.88E+06	14.07%	4.31%
7/1/2015	13	1.70E+06	4.80E+06	4.70E+05	5.60E+04	9.79%	1.17%
Average		2.11E+07	2.65E+07	4.40E+06	1.87E+06	26.88%	18.45%
Standard Error		2.30E+03	2.30E+03	9.38E+02	6.11E+02	6.54%	10.20%
95% Confidence ±		4.50E+03	4.51E+03	1.84E+03	1.20E+03	12.82%	20.00%

Hospital 2 Sewage (Burn Unit, Laboratory, Intensive Care Unit):

Table 2.A – Hospital 2 (Burn, Lab, ICU) *E. coli* Concentration (CFU / 100mL)

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/11/2015	9	No BR	2.33E+04	4.83E+02	2.00E+02	2.07%	0.86%
5/18/2015	10	1.00E+04	2.40E+05	1.01E+04	9.61E+03	4.19%	4.00%
5/26/2015	11	2.33E+05	3.73E+05	1.98E+03	1.70E+03	0.53%	0.46%
6/15/2015	12	1.28E+06	1.43E+06	1.34E+05	3.00E+04	9.33%	2.09%
7/1/2015	13	0.00E+00	1.00E+04	0.00E+00	0.00E+00	0.00%	0.00%
Average		3.79E+05	4.16E+05	2.93E+04	8.30E+03	3.22%	1.48%
Standard Error		3.08E+02	2.88E+02	7.65E+01	4.07E+01	1.69%	0.72%
95% Confidence ±		6.04E+02	5.65E+02	1.50E+02	7.99E+01	3.32%	1.41%

Table 2.B – Hospital 2 (Burn, Lab, ICU) Non-*E. coli* coliforms **Concentration (CFU / 100mL**

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/6/2015	8	3.33E+05	1.00E+06	3.33E+05	0.00E+00	33.33%	0.00%
5/11/2015	9	No BR	1.67E+04	4.17E+02	7.67E+02	2.50%	4.60%
5/18/2015	10	7.45E+05	1.08E+05	6.97E+04	5.83E+04	64.69%	54.07%
5/26/2015	11	2.65E+04	1.90E+04	2.65E+02	9.50E+01	1.39%	0.50%
6/15/2015	12	1.72E+06	2.00E+06	5.70E+05	1.28E+05	28.51%	6.39%
7/1/2015	13	3.00E+04	5.00E+04	0.00E+00	5.00E+04	0.00%	100.00%
Average		5.70E+05	5.32E+05	1.62E+05	3.95E+04	21.74%	27.59%
Standard Error		3.38E+02	2.98E+02	1.64E+02	8.11E+01	10.46%	16.75%
95% Confidence ±		6.62E+02	5.84E+02	3.22E+02	1.59E+02	20.50%	32.83%

Table 2.C – Hospital 2 (Burn, Lab, ICU) Total coliform Concentration (CFU / 100mL)

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/11/2015	9	No BR	4.00E+04	9.00E+02	9.67E+02	2.25%	2.42%
5/18/2015	10	7.55E+05	3.48E+05	7.98E+04	6.79E+04	22.94%	19.52%
5/26/2015	11	2.59E+05	3.92E+05	2.24E+03	1.80E+03	0.57%	0.46%
6/15/2015	12	2.99E+06	3.43E+06	7.04E+05	1.58E+05	20.50%	4.59%
7/1/2015	13	3.00E+04	6.00E+04	0.00E+00	5.00E+04	0.00%	83.33%
Average		1.01E+06	8.55E+05	1.57E+05	5.57E+04	9.25%	22.06%
Standard Error		5.02E+02	4.13E+02	1.77E+02	1.06E+02	5.12%	15.68%
95% Confidence ±		9.84E+02	8.10E+02	3.48E+02	2.07E+02	10.03%	30.74%

Hospital 3 Sewage (Cardiovascular Unit, Intensive Care Unit):

Table 3.A – Hospital 3 (Heart, ICU) *E. coli* Concentration (CFU / 100mL)

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/11/2015	9	No BR	2.22E+04	1.32E+04	6.50E+02	59.60%	2.93%
5/18/2015	10	2.50E+03	7.00E+04	3.83E+04	1.83E+04	54.76%	26.19%
5/26/2015	11	2.15E+07	4.55E+07	1.53E+03	1.55E+03	0.00%	0.00%
6/15/2015	12	1.70E+06	2.25E+06	1.24E+05	7.58E+05	5.52%	33.67%
7/1/2015	13	-	-	1.00E+03	0.00E+00	1.04%	6.33%
Average		7.73E+06	1.20E+07	3.57E+04	1.56E+05	24.19%	13.82%
Standard Error		1.61E+03	1.73E+03	8.45E+01	1.76E+02	13.52%	6.75%
95% Confidence ±		3.15E+03	3.39E+03	1.66E+02	3.46E+02	26.51%	13.24%

**The average Bio-Rad concentration was used for these weeks, due to issues with dilutions, supplies availability, or swarming by protists. These concentrations previously were at zero or counts were not possible*

Table 3.B – Hospital 3 (Heart, ICU) Non- *E. coli* coliforms Concentration (CFU / 100mL)

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/6/2015	8	3.33E+05	6.67E+05	3.33E+05	3.33E+05	50.00%	50.00%
5/11/2015	9	No BR	3.50E+04	2.42E+04	8.10E+03	69.21%	23.14%
5/18/2015	10	2.05E+05	1.03E+05	1.71E+05	1.93E+05	165.95%	187.89%
5/26/2015	11	1.30E+06	1.90E+06	8.05E+02	1.25E+03	0.04%	0.07%
6/15/2015	12	1.40E+07	7.55E+06	2.83E+05	1.38E+06	3.74%	18.21%
7/1/2015	13	0.00E+00	7.00E+04	5.00E+03	0.00E+00	7.14%	0.00%
Average		3.16E+06	1.72E+06	1.36E+05	3.18E+05	49.35%	46.55%
Standard Error		7.95E+02	5.36E+02	1.51E+02	2.30E+02	26.00%	29.25%
95% Confidence ±		1.56E+03	1.05E+03	2.95E+02	4.52E+02	50.96%	57.33%

Table 3.C – Hospital 3 (Heart, ICU) Total coliform Concentration (CFU / 100mL

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/11/2015	9	No BR	5.72E+04	3.75E+04	8.75E+03	65.48%	15.29%
5/18/2015	10	2.07E+05	1.73E+05	2.09E+05	2.11E+05	120.90%	122.38%
5/26/2015	11	2.28E+07	4.74E+07	2.33E+03	2.80E+03	0.00%	0.01%
6/15/2015	12	1.57E+07	9.80E+06	4.07E+05	2.13E+06	4.15%	21.76%
7/1/2015	13	0.00E+00	7.00E+04	6.00E+03	0.00E+00	8.57%	0.00%
Average		9.67E+06	1.15E+07	1.32E+05	4.71E+05	39.82%	31.89%
Standard Error		1.55E+03	1.52E+03	1.63E+02	3.07E+02	23.52%	23.02%
95% Confidence ±		3.05E+03	2.97E+03	3.19E+02	6.02E+02	46.10%	45.12%

Hospital 4 Sewage (Emergency Room and Neurological Unit):

Table 4.A – Hospital 4 (ER, Neuro) *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/6/2015	8	2.88E+07	3.97E+07	2.67E+06	3.33E+05	6.72%	0.84%
5/11/2015	9	No BR	5.11E+05	5.63E+04	2.31E+03	11.02%	0.45%
5/18/2015	10	5.00E+03	3.33E+04	2.75E+04	5.58E+02	82.50%	1.68%
5/26/2015	11	4.95E+05	2.28E+03	5.10E+03	1.00E+02	224.18%	4.40%
6/15/2015	12	5.58E+06	6.73E+06	5.95E+05	6.75E+04	8.85%	1.00%
7/1/2015	13	5.00E+04	1.10E+05	4.00E+04	5.00E+03	36.36%	4.55%
Average		6.98E+06	7.84E+06	5.65E+05	6.81E+04	61.61%	2.15%
Standard Error		1.18E+03	1.14E+03	3.07E+02	1.07E+02	34.57%	0.75%
95% Confidence ±		2.32E+03	2.24E+03	6.02E+02	2.09E+02	67.76%	1.47%

Table 4.B – Hospital 4 (ER, Neuro) Non- *E. coli* coliforms Concentration (CFU / 100mL)

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/6/2015	8	3.17E+06	3.00E+06	3.33E+06	1.00E+06	111.11%	33.33%
5/11/2015	9	No BR	3.00E+05	3.64E+05	3.02E+04	121.30%	10.07%
5/18/2015	10	2.50E+05	1.00E+05	1.24E+05	2.10E+04	124.44%	20.97%
5/26/2015	11	6.13E+05	4.43E+03	7.50E+02	5.02E+02	16.95%	11.34%
6/15/2015	12	4.40E+06	4.57E+07	7.28E+05	1.46E+05	1.59%	0.32%
7/1/2015	13	2.10E+05	6.40E+05	2.90E+05	1.70E+04	45.31%	2.66%
Average		1.73E+06	8.28E+06	8.07E+05	2.02E+05	70.12%	13.11%
Standard Error		5.88E+02	1.29E+03	3.67E+02	1.84E+02	22.65%	5.02%
95% Confidence ±		1.15E+03	2.52E+03	7.19E+02	3.60E+02	44.39%	9.84%

Table 4.C – Hospital 4 (ER, Neuro) Total coliform Concentration (CFU / 100mL)

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/6/2015	8	3.19E+07	4.27E+07	6.00E+06	1.33E+06	14.06%	3.13%
5/11/2015	9	No BR	8.11E+05	4.20E+05	3.25E+04	51.81%	4.01%
5/18/2015	10	2.55E+05	1.33E+05	1.52E+05	2.15E+04	113.96%	16.15%
5/26/2015	11	1.11E+06	6.70E+03	5.85E+03	6.02E+02	87.31%	8.98%
6/15/2015	12	9.98E+06	5.24E+07	1.32E+06	2.14E+05	2.53%	0.41%
7/1/2015	13	2.60E+05	7.50E+05	3.30E+05	2.20E+04	44.00%	2.93%
Average		8.71E+06	1.61E+07	1.37E+06	2.71E+05	52.28%	5.93%
Standard Error		1.32E+03	1.64E+03	4.78E+02	2.12E+02	17.36%	2.34%
95% Confidence ±		2.59E+03	3.21E+03	9.37E+02	4.16E+02	34.02%	4.59%

Raw Sewage:

Table 5.A - Raw Sewage *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	W1	1.89E+06	6.67E+06	7.81E+04	5.56E+03	4.14%	0.29%
2/16/2015	W2	8.33E+05	-	1.50E+05	1.51E+04	18.00%	1.81%
3/2/2015	W3	-	-	8.33E+03	7.25E+03	0.32%	0.28%
3/17/2015	W4	-	-	5.00E+04	7.92E+03	1.94%	0.31%
3/23/2015	W5	3.33E+05	2.67E+06	1.33E+04	8.83E+03	4.00%	2.65%
3/31/2015	W6	1.25E+06	7.92E+06	6.48E+04	4.43E+04	5.18%	3.54%
4/9/2015	W7	2.33E+06	1.52E+07	1.73E+06	3.23E+05	74.29%	13.86%
5/26/2015	W11	5.50E+06	9.00E+06	1.12E+05	1.28E+05	2.03%	2.32%
6/15/2015	W12	3.50E+06	4.00E+07	2.53E+05	5.53E+04	7.21%	1.58%
7/1/2015	W13	5.00E+06	3.00E+06	2.40E+05	4.80E+04	4.80%	0.96%
Average		2.58E+06	1.21E+07	2.70E+05	6.43E+04	12.19%	2.76%
Standard Error		5.68E+02	1.31E+03	1.64E+02	8.02E+01	7.10%	1.30%
95% Confidence ±		1.11E+03	2.57E+03	3.22E+02	1.57E+02	13.90%	2.50%

**The average Bio-Rad concentration was used for these weeks, due to issues with dilutions, supplies availability, or swarming by protists. These concentrations previously were at zero or counts were not possible*

Table 5.B - Raw Sewage Non-*E. coli* coliforms Concentration **(CFU / 100mL) by Media and Date**

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	W1	2.13E+07	4.00E+07	7.48E+05	1.30E+05	3.52%	0.61%
2/16/2015	W2	2.50E+07	2.17E+07	7.08E+05	2.87E+05	2.83%	1.15%
3/2/2015	W3	3.33E+06	6.67E+06	5.25E+05	1.20E+05	15.75%	3.59%
3/17/2015	W4	1.42E+07	3.58E+07	2.83E+05	1.96E+05	2.00%	1.38%
3/23/2015	W5	7.42E+06	1.23E+07	7.42E+04	3.68E+04	1.00%	0.50%
3/31/2015	W6	2.95E+07	2.14E+07	6.88E+05	1.92E+05	2.33%	0.65%
4/9/2015	W7	6.27E+07	1.75E+07	3.72E+06	8.20E+05	5.93%	1.31%
5/26/2015	W11	1.10E+07	1.38E+07	5.38E+05	4.00E+04	4.89%	0.36%
6/15/2015	W12	9.80E+07	3.75E+07	2.32E+06	3.48E+05	2.37%	0.36%
7/1/2015	W13	6.20E+07	4.20E+07	2.11E+06	1.97E+05	3.40%	0.32%
Average		3.34E+07	2.49E+07	1.17E+06	2.37E+05	4.40%	1.02%
Standard Error		1.83E+03	1.58E+03	3.42E+02	1.54E+02	1.3%	0.3%
95% Confidence ±		3.58E+03	3.09E+03	6.71E+02	3.02E+02	2.6%	0.6%

Table 5.C - Raw Sewage Total coliform Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	W1	2.32E+07	4.67E+07	8.26E+05	1.36E+05	3.57%	0.59%
2/16/2015	W2	2.58E+07	-	8.58E+05	3.02E+05	3.32%	1.17%
3/2/2015	W3	-	-	5.33E+05	1.27E+05	1.26%	0.30%
3/17/2015	W4	-	-	3.33E+05	2.04E+05	0.79%	0.48%
3/23/2015	W5	7.75E+06	1.50E+07	8.75E+04	4.56E+04	1.13%	0.59%
3/31/2015	W6	3.08E+07	2.93E+07	7.52E+05	2.37E+05	2.45%	0.77%
4/9/2015	W7	6.50E+07	3.27E+07	5.45E+06	1.14E+06	8.38%	1.76%
5/26/2015	W11	1.65E+07	2.28E+07	6.50E+05	1.68E+05	3.94%	1.02%
6/15/2015	W12	1.02E+08	7.75E+07	2.58E+06	4.03E+05	2.54%	0.40%
7/1/2015	W13	6.70E+07	4.50E+07	2.35E+06	2.45E+05	3.51%	0.37%
Average		4.22E+07	3.84E+07	1.44E+06	3.01E+05	3.09%	0.74%
Standard Error		2.30E+03	2.34E+03	3.80E+02	1.73E+02	0.7%	0.1%
95% Confidence ±		4.50E+03	4.59E+03	7.44E+02	3.40E+02	1.3%	0.3%

Secondary Effluent:

Table 6.A – Secondary Effluent *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	W1	2.11E+03	2.06E+04	7.52E+01	1.44E+01	3.56%	0.68%
2/16/2015	W2	1.25E+04	2.50E+04	8.33E+01	7.50E+01	0.67%	0.60%
3/2/2015	W3	6.67E+03	3.33E+04	1.17E+02	2.92E+01	1.75%	0.44%
3/17/2015	W4	1.67E+03	0.00E+00	3.33E+01	1.50E+01	2.00%	0.90%
3/23/2015	W5	1.42E+03	4.25E+03	4.67E+01	2.33E+01	3.29%	1.65%
3/31/2015	W6	7.50E+04	7.42E+04	2.97E+02	1.23E+02	0.40%	0.16%
4/9/2015	W7	6.67E+03	1.03E+05	9.50E+02	2.67E+02	14.25%	4.00%
5/26/2015	W11	2.55E+05	1.98E+03	4.00E+01	0.00E+00	0.02%	0.00%
6/15/2015	W12	2.15E+04	4.53E+04	6.53E+02	8.75E+01	3.03%	0.41%
7/1/2015	W13	1.00E+04	3.00E+04	6.00E+02	9.00E+02	6.00%	9.00%
Average		3.93E+04	3.38E+04	2.89E+02	1.53E+02	3.50%	1.78%
Standard Error		6.27E+01	5.81E+01	5.38E+00	3.92E+00	1.3%	0.9%
95% Confidence ±		1.23E+02	1.14E+02	1.05E+01	7.68E+00	2.6%	1.7%

Table 6.B – Secondary Effluent Non-*E. coli* coliforms Concentration **(CFU / 100mL) by Media and Date**

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	3.28E+04	9.33E+04	2.72E+02	3.67E+02	0.83%	1.12%
2/16/2015	2	7.08E+04	1.46E+05	0.00E+00	9.50E+02	0.00%	1.34%
3/2/2015	3	1.25E+04	2.67E+05	3.83E+02	5.65E+02	3.07%	4.52%
3/17/2015	4	1.08E+04	2.67E+04	2.83E+02	1.61E+02	2.62%	1.48%
3/23/2015	5	2.01E+04	1.95E+04	2.55E+02	2.01E+02	1.27%	1.00%
3/31/2015	6	1.39E+05	1.31E+05	1.88E+03	1.42E+03	1.35%	1.02%
4/9/2015	7	2.18E+05	9.00E+04	4.88E+03	3.28E+03	2.24%	1.50%
5/26/2015	11	2.05E+03	9.25E+02	6.75E+01	3.50E+01	3.29%	1.71%
6/15/2015	12	2.22E+05	1.10E+05	1.53E+03	5.65E+02	0.69%	0.25%
7/1/2015	13	2.30E+05	1.40E+05	1.50E+03	3.50E+03	0.65%	1.52%
Average		9.58E+04	1.02E+05	1.11E+03	1.10E+03	1.60%	1.55%
Standard Error		9.79E+01	1.01E+02	1.05E+01	1.05E+01	0.4%	0.4%
95% Confidence ±		1.92E+02	1.98E+02	2.06E+01	2.06E+01	0.7%	0.7%

Table 6.C – Secondary Effluent Total coliform Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	3.49E+04	1.14E+05	3.47E+02	3.81E+02	1.00%	1.09%
2/16/2015	2	8.33E+04	1.71E+05	8.33E+01	1.03E+03	0.10%	1.23%
3/2/2015	3	1.92E+04	3.00E+05	5.00E+02	5.94E+02	2.61%	3.10%
3/17/2015	4	1.25E+04	2.67E+04	3.17E+02	1.76E+02	2.53%	1.41%
3/23/2015	5	2.15E+04	2.38E+04	3.02E+02	2.24E+02	1.40%	1.04%
3/31/2015	6	2.14E+05	2.05E+05	2.18E+03	1.54E+03	1.02%	0.72%
4/9/2015	7	2.25E+05	1.93E+05	5.83E+03	3.55E+03	2.59%	1.58%
5/26/2015	11	2.57E+05	2.90E+03	1.08E+02	3.50E+01	0.04%	0.01%
6/15/2015	12	2.43E+05	1.56E+05	2.18E+03	6.53E+02	0.90%	0.27%
7/1/2015	13	2.40E+05	1.70E+05	2.10E+03	4.40E+03	0.88%	1.83%
Average		1.35E+05	1.36E+05	1.39E+03	1.26E+03	1.31%	1.23%
Standard Error		1.16E+02	1.17E+02	1.18E+01	1.12E+01	0.3%	0.3%
95% Confidence ±		2.28E+02	2.29E+02	2.31E+01	2.20E+01	0.6%	0.5%

Morgan Creek Upstream:

Table 7.A – Morgan Creek Upstream *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	1.94E+02	6.11E+02	0.00E+00	0.00E+00	0.00%	0.00%
2/16/2015	2	0.00E+00	3.33E+02	0.00E+00	0.00E+00	0.00%	0.00%
3/2/2015	3	8.25E+01	6.33E+02	3.33E+00	8.33E+00	0.53%	1.32%
3/17/2015	4	1.83E+02	3.33E+01	5.00E+00	0.00E+00	15.00%	0.00%
3/23/2015	5	1.00E+02	2.50E+02	1.67E+00	0.00E+00	0.67%	0.00%
3/31/2015	6	1.17E+02	7.08E+03	0.00E+00	0.00E+00	0.00%	0.00%
4/9/2015	7	3.33E+01	4.00E+02	3.33E+01	0.00E+00	8.33%	0.00%
5/26/2015	11	1.38E+02	2.90E+02	0.00E+00	0.00E+00	0.00%	0.00%
6/15/2015	12	1.73E+02	8.50E+02	5.00E+00	5.00E+00	0.59%	0.59%
7/1/2015	13	3.00E+03	3.00E+03	2.00E+02	9.00E+02	6.67%	30.00%
Average		4.02E+02	1.35E+03	2.48E+01	9.13E+01	3.18%	3.19%
Standard Error		6.34E+00	1.16E+01	1.58E+00	3.02E+00	1.6%	3.0%
95% Confidence ±		1.24E+01	2.28E+01	3.09E+00	5.92E+00	3.2%	5.8%

Table 7.B – Morgan Creek Upstream Non-*E. coli* coliforms Concentration **(CFU / 100mL) by Media and Date**

Date	Week	Bio-Rad Rapid' ***E. coli 2*** Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL coliforms	% KPC coliform
2/9/2015	1	9.15E+02	1.44E+03	2.00E+01	0.00E+00	2.19%	0.00%
2/16/2015	2	2.33E+03	9.25E+03	3.33E+00	5.83E+00	0.14%	0.25%
3/2/2015	3	4.03E+02	7.25E+02	4.83E+01	2.33E+01	11.98%	5.79%
3/17/2015	4	1.46E+03	1.03E+03	4.17E+01	5.00E+00	2.86%	0.34%
3/23/2015	5	2.37E+03	1.04E+03	2.17E+01	5.00E+01	0.92%	2.11%
3/31/2015	6	4.03E+03	1.14E+04	3.33E+00	1.33E+01	0.08%	0.33%
4/9/2015	7	6.82E+03	3.00E+02	1.00E+02	0.00E+00	1.47%	0.00%
5/26/2015	11	1.23E+02	3.90E+02	0.00E+00	0.00E+00	0.00%	0.00%
6/15/2015	12	1.12E+04	6.60E+03	3.00E+01	2.95E+02	0.27%	2.63%
7/1/2015	13	1.22E+05	4.40E+04	1.50E+03	3.50E+03	1.23%	2.87%
Average		1.52E+04	7.62E+03	1.77E+02	3.89E+02	2.11%	1.43%
Standard Error		3.89E+01	2.76E+01	4.21E+00	6.24E+00	1.1%	0.6%
95% Confidence ±		7.63E+01	5.41E+01	8.24E+00	1.22E+01	2.2%	1.2%

Table 7.C – Morgan Creek Upstream Total coliform Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	1.11E+03	2.06E+03	2.00E+01	0.00E+00	1.80%	0.00%
2/16/2015	2	2.33E+03	9.58E+03	3.33E+00	5.83E+00	0.14%	0.25%
3/2/2015	3	4.86E+02	1.36E+03	5.17E+01	3.17E+01	10.63%	6.52%
3/17/2015	4	1.64E+03	1.07E+03	4.67E+01	5.00E+00	2.84%	0.30%
3/23/2015	5	2.47E+03	1.29E+03	2.33E+01	5.00E+01	0.95%	2.03%
3/31/2015	6	4.14E+03	1.85E+04	3.33E+00	1.33E+01	0.08%	0.32%
4/9/2015	7	6.85E+03	7.00E+02	1.33E+02	0.00E+00	1.95%	0.00%
5/26/2015	11	2.60E+02	6.80E+02	0.00E+00	0.00E+00	0.00%	0.00%
6/15/2015	12	1.14E+04	7.45E+03	3.50E+01	3.00E+02	0.31%	2.63%
7/1/2015	13	1.25E+05	4.70E+04	1.70E+03	4.40E+03	1.36%	3.52%
Average		1.56E+04	8.97E+03	2.02E+02	4.81E+02	2.01%	1.56%
Standard Error		3.95E+01	2.99E+01	4.49E+00	6.93E+00	1.0%	0.7%
95% Confidence ±		7.73E+01	5.87E+01	8.80E+00	1.36E+01	2.0%	1.3%

Morgan Creek Downstream:

Table 8.A – Morgan Creek Downstream *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	8.06E+01	1.33E+03	7.78E+00	0.00E+00	9.66%	0.00%
2/16/2015	2	1.67E+02	1.08E+03	3.33E+00	0.00E+00	2.00%	0.00%
3/2/2015	3	9.00E+03	6.25E+04	5.00E+00	0.00E+00	0.06%	0.00%
3/17/2015	4	6.67E+01	0.00E+00	3.33E+00	0.00E+00	5.00%	0.00%
3/23/2015	5	6.67E+01	4.08E+02	6.67E+00	1.67E+00	10.00%	2.50%
3/31/2015	6	8.33E+01	6.67E+03	0.00E+00	0.00E+00	0.00%	0.00%
4/9/2015	7	1.00E+02	1.27E+03	6.67E+01	0.00E+00	66.67%	0.00%
5/26/2015	11	1.45E+02	1.73E+02	0.00E+00	0.00E+00	0.00%	0.00%
6/15/2015	12	1.83E+02	1.25E+03	1.00E+01	3.00E+01	5.48%	16.44%
7/1/2015	13	3.00E+03	4.00E+03	5.00E+02	0.00E+00	16.67%	0.00%
Average		1.29E+03	7.87E+03	6.03E+01	3.17E+00	11.55%	1.89%
Standard Error		1.14E+01	2.81E+01	2.46E+00	5.63E-01	6.4%	1.6%
95% Confidence ±		2.23E+01	5.50E+01	4.81E+00	1.10E+00	12.5%	3.2%

Table 8.B – Morgan Creek Downstream Non-*E. coli* coliforms Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid'*E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	8.61E+02	8.89E+02	3.11E+01	0.00E+00	3.61%	0.00%
2/16/2015	2	1.17E+03	9.50E+03	5.00E+00	1.33E+01	0.43%	1.14%
3/2/2015	3	2.31E+05	7.08E+04	4.92E+01	9.17E+01	0.02%	0.04%
3/17/2015	4	1.54E+03	7.67E+02	3.75E+01	1.17E+01	2.43%	0.76%
3/23/2015	5	3.23E+03	1.83E+03	9.58E+01	1.00E+01	2.96%	0.31%
3/31/2015	6	3.08E+03	8.83E+03	3.33E+00	1.00E+01	0.11%	0.33%
4/9/2015	7	6.78E+03	3.47E+03	2.33E+02	1.67E+01	3.44%	0.25%
5/26/2015	11	1.55E+02	5.53E+02	0.00E+00	0.00E+00	0.00%	0.00%
6/15/2015	12	8.60E+03	8.45E+03	4.50E+01	3.65E+02	0.52%	4.24%
7/1/2015	13	5.60E+04	4.50E+04	3.20E+03	9.00E+02	5.71%	1.61%
Average		3.12E+04	1.50E+04	3.70E+02	1.42E+02	1.92%	0.87%
Standard Error		5.59E+01	3.87E+01	6.08E+00	3.77E+00	0.63%	0.41%
95% Confidence ±		1.10E+02	7.59E+01	1.19E+01	7.38E+00	1.23%	0.81%

Table 8.C – Morgan Creek Downstream Total coliform Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid'*E. coli 2* Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
2/9/2015	1	9.42E+02	2.22E+03	3.89E+01	0.00E+00	4.13%	0.00%
2/16/2015	2	1.33E+03	1.06E+04	8.33E+00	1.33E+01	0.63%	1.00%
3/2/2015	3	2.40E+05	1.33E+05	5.42E+01	9.17E+01	0.02%	0.04%
3/17/2015	4	1.61E+03	7.67E+02	4.08E+01	1.17E+01	2.54%	0.73%
3/23/2015	5	3.30E+03	2.24E+03	1.03E+02	1.17E+01	3.11%	0.35%
3/31/2015	6	3.16E+03	1.55E+04	3.33E+00	1.00E+01	0.11%	0.32%
4/9/2015	7	6.88E+03	4.73E+03	3.00E+02	1.67E+01	4.36%	0.24%
5/26/2015	11	3.00E+02	7.25E+02	0.00E+00	0.00E+00	0.00%	0.00%
6/15/2015	12	8.78E+03	9.70E+03	5.50E+01	3.95E+02	0.63%	4.50%
7/1/2015	13	5.90E+04	4.90E+04	3.70E+03	9.00E+02	6.27%	1.53%
Average		3.25E+04	2.29E+04	4.30E+02	1.45E+02	2.18%	0.87%
Standard Error		5.70E+01	4.78E+01	6.56E+00	3.81E+00	0.71%	0.43%
95% Confidence ±		1.12E+02	9.38E+01	1.29E+01	7.46E+00	1.38%	0.85%

Jordan Lake:

Table 9.A – Jordan Lake *E. coli* Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/26/2015	11	0	82.5	0	1.67	0.00%	2.02%
6/15/2015	12	425	750	5		0.67%	0.00%
7/1/2015	13	0	13000	100	0	0.77%	0.00%
Average		1.42E+02	4.61E+03	3.50E+01	8.33E-01	0.48%	0.67%
Standard Error		6.87E+00	3.92E+01	3.42E+00	6.45E-01	0.2%	0.7%
95% Confidence ±		1.35E+01	7.68E+01	6.69E+00	1.27E+00	0.5%	1.3%

Table 9.B – Jordan Lake Non- *E. coli* coliforms Concentration **(CFU / 100mL) by Media and Date**

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/26/2015	11	15	132.5	0	503.75	0.00%	380.19%
6/15/2015	12	24500	22175	5		0.02%	0.00%
7/1/2015	13	50000	48000	1200	600	2.50%	1.25%
Average		2.48E+04	2.34E+04	4.02E+02	5.52E+02	0.84%	127.15%
Standard Error		9.10E+01	8.84E+01	1.16E+01	1.66E+01	0.8%	126.5%
95% Confidence ±		1.78E+02	1.73E+02	2.27E+01	3.26E+01	1.6%	248.0%

Table 9.C – Jordan Lake Total coliform Concentration (CFU / 100mL) by Media and Date

Date	Week	Bio-Rad Rapid' *E. coli* 2 Agar	CHROMagar Orientation	CHROMagar ESBL	CHROMagar KPC	% ESBL	% KPC
5/26/2015	11	15	215	0	505.42	0.00%	235.08%
6/15/2015	12	24925	22925	10		0.04%	0.00%
7/1/2015	13	50000	61000	1300	600	2.13%	0.98%
Average		2.50E+04	2.80E+04	4.37E+02	5.53E+02	0.72%	78.69%
Standard Error		9.13E+01	9.67E+01	1.21E+01	1.66E+01	0.7%	78.2%
95% Confidence ±		1.79E+02	1.90E+02	2.36E+01	3.26E+01	1.4%	153.3%

APPENDIX 3 – ESBL MALDI-TOF MS AND VITEK 2 RESULTS

S = Susceptible I = Intermediate R = Resistant based on MIC R = Resistant based on other phenotypic properties (VITEK Software Determined)*

Pos = Positive ESBL Production Neg = Negative for ESBL Production (.) = Not applicable

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
AE09	3	Raw Sewage	ECOLI	1	*Klebsiella oxytoca*	1	>=8	R	POS	4	R*
AE10	3	Raw Sewage	ECOLI	1	*Klebsiella oxytoca*	1	>=8	R	POS	8	R*
AE12	4	Raw Sewage	ECOLI	1	*Escherichia coli*	0	.	.	.	.	.
AE16	5	Raw Sewage	ECOLI	1	*Stenotrophomonas maltophilia*	1	Not clinically significant				
AE18	6	Raw Sewage	ECOLI	1	*Streptococcus cristatus*	0	.	.	.	.	.
AE19	6	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE20	6	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE23	7	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE24	7	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE25	7	Raw Sewage	ECOLI	1	*Staphylococcus saprophyticus*	0	.	.	.	.	.
AE26	11	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE27	11	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE28	11	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE29	12	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	NEG	<=1	S
AE31	12	Raw Sewage	ECOLI	1	*Citrobacter freundii*	1	>=8	R		4	R*
AE32	12	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE33	12	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE34	13	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AE36	13	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	NEG	<=1	R*
AE37	13	Raw Sewage	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AT09	3	Raw Sewage	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	POS	4	R*
AT10	3	Raw Sewage	KLEBSIELLA	1	*Citrobacter werkmanii*	1	>=8	R		8	I
AT11	3	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	I
AT13	4	Raw Sewage	KLEBSIELLA	1	*Serratia marcescens*	1				2	S

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
AT15	4	Raw Sewage	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		8	I
AT16	4	Raw Sewage	KLEBSIELLA	1	*Citrobacter farmeri*	1	>=8	R		<=1	S
AT17	5	Raw Sewage	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	1	>=8	R		<=1	S
AT18	5	Raw Sewage	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		8	I
AT20	5	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT21	5	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	I
AT22	6	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	4	R*
AT23	6	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		2	S
AT24	6	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*
AT25	6	Raw Sewage	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	1	2	S		<=1	S
AT27	7	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*
AT28	7	Raw Sewage	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	1	2	S		<=1	S
AT29	7	Raw Sewage	KLEBSIELLA	1	*Pseudomonas putida*	1	>=8	R		<=1	S
AT31	7	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	4	R*
AT32	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT32 - Turq	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*
AT33	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT34	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT34 -Turq	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT35	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT36	11	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	I
AT37	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	>=16	R
AT38	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT39	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	2	R*
AT40	12	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	4	I		2	S

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
AT41	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
AT42	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT42 - Turq	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT43	12	Raw Sewage	KLEBSIELLA	1	*Pseudomonas aeruginosa*	1	>=8	R		<=1	S
AT44	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
AT44 pink (A)	12	Raw Sewage	KLEBSIELLA	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
AT45	13	Raw Sewage	KLEBSIELLA	1	*Raoultella planticola*	1	>=8	R		4	S
AT46	13	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*
AT47	13	Raw Sewage	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		8	I
AT48	13	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	I
AT49 - Small	13	Raw Sewage	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		>=16	R
AT49 large	13	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	R
BE07	3	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE08	3	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE09	3	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE10 - turq	4	Secondary Effluent	ECOLI	1	*Aeromonas hydrophila / caviae*	1	>=8	R		8	I
BE11	5	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE12	5	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE13	5	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE14	5	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE15	5	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE16	6	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
BE17	6	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE18	6	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE19	6	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE20	6	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE21	7	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE22	7	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE23	7	Secondary Effluent	ECOLI	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BE23 - TURQ	7	Secondary Effluent	ECOLI	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BE24	7	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE25	7	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE26	11	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE27	11	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE29	12	Secondary Effluent	ECOLI	1	*Stenotrophomonas maltophilia*	1		Not clinically significant			
BE30	12	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE31	12	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE32	12	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BE33	13	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	>=8	R		<=1	S
BE34	13	Secondary Effluent	ECOLI	1	*Streptococcus parasanguinis*	0	.	.	.	.	.
BE35	13	Secondary Effluent	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BT11	3	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*
BT11 - Turq	3	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
BT12	3	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	I
BT13	3	Secondary Effluent	KLEBSIELLA	1	*Serratia odorifera/fonticola*	1				<=1	S
BT13 - dup	3	Secondary Effluent	KLEBSIELLA	1	*Serratia odorifera/fonticola*	0	.	.	.	.	.
BT16	4	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		4	S
BT17	4	Secondary Effluent	KLEBSIELLA	1	*Serratia marcescens*	1				>=16	R
BT18	4	Secondary Effluent	KLEBSIELLA	1	*Citrobacter freundii / werkmanii*	1	>=8	R		8	I
BT19	4	Secondary Effluent	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		8	I
BT20	4	Secondary Effluent	KLEBSIELLA	1	*Aeromonas sobria / hydrophila / caviae*	1	>=8	R		<=1	S
BT21	5	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
BT22	5	Secondary Effluent	KLEBSIELLA	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
BT23	5	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	8	R*
BT24	5	Secondary Effluent	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		2	S
BT26	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
BT27	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
BT28	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BT28 - Turq	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BT29	7	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
BT30	7	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	4	R*
BT31	7	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BT32	7	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
BT33	11	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
BT34	11	Secondary Effluent	KLEBSIELLA	1	*Cronobacter sakazakii*	1	>=8	R		4	S
BT35	11	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BT36	11	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	8	R*
BT37	11	Secondary Effluent	KLEBSIELLA	1	*Cronobacter sakazakii*	1	>=8	R		>=16	R
BT38	11	Secondary Effluent	KLEBSIELLA	1	*Citrobacter freundii*	1	4	R*		8	R*
BT40	12	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	4	I		4	S
BT41	12	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	8	R*
BT42	12	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	4	R*	POS	<=1	R*
BT43	12	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	R*
BT45	13	Secondary Effluent	KLEBSIELLA	1	*Serratia fonticola*	1				<=1	S
BT46	13	Secondary Effluent	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	>=8	R		8	I
BT47	13	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
CE01	3	Morgan Creek Upstream	ECOLI	1	*Serratia fonticola*	0	.	.	.	.	.
CE02	5	Morgan Creek Upstream	ECOLI	1	*Serratia fonticola*	0	.	.	.	.	.
CE03	6	Morgan Creek Upstream	ECOLI	1	*Citrobacter freundii*	0	.	.	.	.	.
FE04	9	H1 GI + MICU	ECOLI	1	*Citrobacter freundii / braaki*	0	.	.	.	.	.
FE06	9	H1 GI + MICU	ECOLI	1	*Citrobacter farmeri / amalonaticus*	0	.	.	.	.	.
FE07	9	H1 GI + MICU	ECOLI	1	*Aeromonas sobria / hydrophila / caviae*	0	.	.	.	.	.
FE08	10	H1 GI + MICU	ECOLI	1	*Ochrobactrum anthropi*	0	.	.	.	.	.
FE09	10	H1 GI + MICU	ECOLI	1	*Citrobacter freundii*	0	.	.	.	.	.
FE10	13	H1 GI + MICU	ECOLI	1	*Citrobacter freundii*	0	.	.	.	.	.
FE12	13	H1 GI + MICU	ECOLI	1	*Citrobacter freundii / braaki*	0	.	.	.	.	.
FE13	13	H1 GI + MICU	ECOLI	1	*Citrobacter freundii*	0	.	.	.	.	.

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
FT01	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella oxytoca*	0	.	.	.	.	.
FT02	8	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter werkmanii*	0	.	.	.	.	.
FT03	8	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT04	8	H1 GI + MICU	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	0	.	.	.	.	.
FT05	8	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT06	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT07	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT08	9	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT09	9	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii / werkmanii*	0	.	.	.	.	.
FT10	9	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT11	9	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT12	9	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT13	10	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT14	10	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter youngae / farmeri*	0	.	.	.	.	.
FT15	10	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	0	.	.	.	.	.
FT16	10	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT18	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT19	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT20	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT21	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT22	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT23	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT24	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT25	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT26	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT27	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
FT28	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT29	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT30	11	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT32	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT33	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT34	12	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
FT35	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT36	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT37	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT38	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT39	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT40	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
FT41	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	0	.	.	.	.	.
GE04	10	H2 Burn + Lab + ICU	ECOLI	1	*Ochrobactrum anthropi*	0	.	.	.	.	.
GE05	10	H2 Burn + Lab + ICU	ECOLI	1	*Ochrobactrum anthropi*	0	.	.	.	.	.
GE06	10	H2 Burn + Lab + ICU	ECOLI	1	*Citrobacter youngae*	0	.	.	.	.	.
GE08	12	H2 Burn + Lab + ICU	ECOLI	1	*Pseudomonas putida*	0	.	.	.	.	.
GE10	13	H2 Burn + Lab + ICU	ECOLI	1	*Serratia rubidaea / marcescens*	0	.	.	.	.	.
HE04	9	H3 Heart + ICU	ECOLI	1	*Citrobacter freundii*	0	.	.	.	.	.
HE05	9	H3 Heart + ICU	ECOLI	1	*Citrobacter freundii*	0	.	.	.	.	.
HE06	10	H3 Heart + ICU	ECOLI	1	*Ochrobactrum anthropi*	0	.	.	.	.	.
HE10	10	H3 Heart + ICU	ECOLI	1	*Ochrobactrum anthropi*	0	.	.	.	.	.
HE12	11	H3 Heart + ICU	ECOLI	1	*Escherichia coli*	0	.	.	.	.	.
HE13	12	H3 Heart + ICU	ECOLI	1	*Citrobacter amalonaticus*	0	.	.	.	.	.

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
HE14	12	H3 Heart + ICU	ECOLI	1	*Citrobacter youngae / farmeri*	0	.	.	.	.	.
HT01	8	H3 Heart + ICU	KLEBSIELLA	1	*Raoultella ornithinolytica*	1	>=8	R		<=1	S
HT02	8	H3 Heart + ICU	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	POS	<=1	R*
HT03	9	H3 Heart + ICU	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	POS	<=1	R*
IE01	8	H4 ER + Neuro	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
IE05	8	H4 ER + Neuro	ECOLI	1	*Citrobacter braakii / werkmanii*	1	TRM			8	R*
IE06	9	H4 ER + Neuro	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
IE07	9	H4 ER + Neuro	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
IE09	10	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		<=1	S
IE10	10	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii / werkmanii*	1	>=8	R		>=16	R
IE11	10	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		2	S
IE12	10	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		8	R*
IE13	10	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		8	R*
IE15	12	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		2	S
IE16	12	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		8	R*
IE17	12	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		4	R*
IE18	12	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		<=1	S
IE21	13	H4 ER + Neuro	ECOLI	1	*Escherichia coli*	1	>=8	R	POS	<=1	R*
IE23	13	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	>=8	R		8	R*
IT01	8	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	NEG	4	R*
IT02	8	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	0	.	.	.	.	.
IT03	8	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R		8	R*

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
IT04	8	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	1	R*	POS	<=1	S
IT05	8	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	NEG	8	R*
IT06	9	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	NEG	>=16	R
IT07	9	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	S
IT08	9	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	POS	<=1	S
IT09	9	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	2	S	NEG	<=1	S
IT12	9	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	2	R*	POS	<=1	S
IT14	9	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	2	S	NEG	<=1	S
IT17	10	H4 ER + Neuro	KLEBSIELLA	1	*Raoultella ornithinolytica*	1	>=8	R		8	I
IT18	10	H4 ER + Neuro	KLEBSIELLA	1	*Citrobacter farmeri*	1	>=8	R		4	S
IT19	11	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		8	I
IT20	11	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	>=8	R		>=16	R
IT21	11	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	S
IT22	12	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	2	R*	POS	<=1	S
IT23	12	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	S
IT23 A	12	H4 ER + Neuro	KLEBSIELLA	1	*Serratia marcescens*	1				>=16	R
IT24	12	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	S
IT26	12	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	>=8	R	POS	<=1	S
IT27	13	H4 ER + Neuro	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	>=8	R		8	I
IT28	13	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	POS	<=1	R*
IT29	13	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	>=8	R	POS	<=1	R*
IT30	13	H4 ER + Neuro	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	>=8	R		<=1	S

ESBL Code	Week	Site	ESBL ID	MALDI -TOF	MALDI TOF Result	NCSU VITEK 2	Cefpodoxime		ESBL	Imipenem	
							MIC	S/I/R	MIC	MIC	S/I/R
IT30-MALDI extra	13	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	2	R*	POS	<=1	S
IT31	13	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	2	R*	POS	<=1	S

APPENDIX 4 – KPC MALDI-TOF MS AND MODIFIED HODGE TEST ISOLATE RESULTS

KPC(+) = Positive KPC Production KPC(R-) = "Intermediate" KPC production KPC(-) = Negative for KPC Production (.) = Not applicable

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
JE11	3	Raw Sewage	ECOLI	1	*Enterobacter cloacae / asburiae*	1	1	0	0
JE12	3	Raw Sewage	ECOLI	1	*Klebsiella pneumoniae*	1	1	0	0
JE13	3	Raw Sewage	ECOLI	0	NO MALDI	1	0	1	0
JE14	3	Raw Sewage	ECOLI	1	*Enterobacter cloacae / asburiae*	1	1	0	0
JE16	4	Raw Sewage	ECOLI	1	*Enterococcus faecium*	1	0	0	1
JE17	4	Raw Sewage	ECOLI	1	*Citrobacter freundii*	1	1	0	0
JE19	5	Raw Sewage	ECOLI	1	*Enterobacter cloacae / asburiae*	1	1	0	0
JE20	5	Raw Sewage	ECOLI	1	*Citrobacter freundii*	1	1	0	0
JE21	6	Raw Sewage	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
JE26	12	Raw Sewage	ECOLI	1	*Serratia marcescens*	1	0	1	0
JE27	12	Raw Sewage	ECOLI	1	*Citrobacter freundii*	1	1	0	0
JE28	12	Raw Sewage	ECOLI	0	NO MALDI	1	1	0	0
JE29	12	Raw Sewage	ECOLI	1	*Pseudomonas aeruginosa*	1	0	1	0
JE30	13	Raw Sewage	ECOLI	0	NO MALDI	1	1	0	0
JE31	13	Raw Sewage	ECOLI	0	NO MALDI	1	1	0	0
JE32	13	Raw Sewage	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
JT11	3	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT12	3	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT13	3	Raw Sewage	KLEBSIELLA	1	*Klebsiella oxytoca*	1	1	0	0
JT14	3	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT15	3	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT17	4	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT18	4	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT19	4	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT19B	4	Raw Sewage	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	1	1	0	0
JT20	4	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	0	1	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
JT22	4	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT23	5	Raw Sewage	KLEBSIELLA	1	*Serratia marcescens*	1	0	0	1
JT24	5	Raw Sewage	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
JT25	5	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT26	5	Raw Sewage	KLEBSIELLA	1	*Serratia marcescens*	1	0	0	1
JT27	5	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
JT28	5	Raw Sewage	KLEBSIELLA	1	*Klebsiella oxytoca*	1	1	0	0
JT29	5	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT30	5	Raw Sewage	KLEBSIELLA	1	*Klebsiella oxytoca*	1	0	1	0
JT31	6	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
JT32	6	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT33	6	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT34	6	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT35	6	Raw Sewage	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	0	1
JT36	11	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
JT37	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	0	1	0
JT38	11	Raw Sewage	KLEBSIELLA	1	*Cronobacter sakazakii*	1	1	0	0
JT39	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT40	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT41	11	Raw Sewage	KLEBSIELLA	1	*Cronobacter sakazakii*	1	1	0	0
JT42	11	Raw Sewage	KLEBSIELLA	1	*Cronobacter sakazakii*	1	1	0	0
JT43	11	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT44	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT45	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT46	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT47	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT48	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT49	12	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT50	13	Raw Sewage	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
JT51	13	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	0	0	1
JT52	13	Raw Sewage	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
JT53	13	Raw Sewage	KLEBSIELLA	1	*Aeromonas hydrophila / caviae*	1	1	0	0
KE09	3	Secondary Effluent	ECOLI	1	*Citrobacter werkmanii*	1	1	0	0
KE11	3	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	1	0	0
KE12	3	Secondary Effluent	ECOLI	1	*Citrobacter farmeri*	1	1	0	0
KE14	4	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	1	0	0
KE16	4	Secondary Effluent	ECOLI	0	NO MALDI	1	1	0	0
KE17A	4	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	1	0	0
KE17B	4	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	1	0	0
KE21	5	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	1	0	0
KE22	5	Secondary Effluent	ECOLI	1	*Pseudomonas putida*	1	1	0	0
KE23	6	Secondary Effluent	ECOLI	1	*Klebsiella pneumoniae*	1	0	1	0
KE24	6	Secondary Effluent	ECOLI	1	*Aeromonas hydrophila / caviae*	1	1	0	0
KE25	6	Secondary Effluent	ECOLI	1	*Stenotrophomonas maltophilia*	1	1	0	0
KE26	6	Secondary Effluent	ECOLI	1	*Citrobacter freundii*	1	1	0	0
KE27	12	Secondary Effluent	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
KE29	13	Secondary Effluent	ECOLI	1	*Enterobacter cloacae / asburiae*	1	1	0	0
KE30	13	Secondary Effluent	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
KE31	13	Secondary Effluent	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
KT11	3	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT12	3	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
KT13	3	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT14	3	Secondary Effluent	KLEBSIELLA	1	*Pantoea aggiomerans*	1	1	0	0
KT15	4	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
KT16	4	Secondary Effluent	KLEBSIELLA	1	*Escherichia coli*	1	0	0	1
KT17	4	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT19	4	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	0	0	1
KT20	5	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
KT21	5	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
KT22	5	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
KT23	5	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT24	5	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	0	1	0
KT25	5	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT26	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT27	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT28	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT29	6	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
KT30	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT31	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT32	6	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT33	12	Secondary Effluent	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
KT34	12	Secondary Effluent	KLEBSIELLA	1	*Klebsiella oxytoca*	1	0	1	0
KT35	12	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	0	1	0
KT35A	12	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	0	1	0
KT36	12	Secondary Effluent	KLEBSIELLA	1	*Klebsiella oxytoca*	1	0	1	0
KT37	12	Secondary Effluent	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
KT40	12	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
KT41	13	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
KT42	13	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	0	0	1
KT43	13	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	0	0	1
KT45	13	Secondary Effluent	KLEBSIELLA	0	NO MALDI	1	1	0	0
LT02	5	Morgan Creek Upstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0
LT03	6	Morgan Creek Upstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	0	.	.	.
LT04	6	Morgan Creek Upstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0
LT05	6	Morgan Creek Upstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
LT06	7	Morgan Creek Upstream	KLEBSIELLA	1	*Bacillus cereus / thuringiensis*	1	0	1	0
LT07	7	Morgan Creek Upstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0
LT09	7	Morgan Creek Upstream	KLEBSIELLA	1	*Staphlycoccus waneri*	1	0	0	1
LT10	12	Morgan Creek Upstream	KLEBSIELLA	1	*Sphingobacterium multivorum*	1	0	0	1
LT11	11	Morgan Creek Upstream	KLEBSIELLA	1	*Sphingobacterium multivorum*	1	0	1	0
MT04	3	Morgan Creek Downstream	KLEBSIELLA	1	*Pseudomonas chororaphis / fluorescens*	1	0	1	0
MT05	3	Morgan Creek Downstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	0	1
MT06	3	Morgan Creek Downstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0
MT07	3	Morgan Creek Downstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	0	1
MT08	4	Morgan Creek Downstream	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	1	0
MT09	6	Morgan Creek Downstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	0	1
MT10	7	Morgan Creek Downstream	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0
MT11	12	Morgan Creek Downstream	KLEBSIELLA	1	*Elizabethkingia meningoseptica*	1	0	1	0
MT12	12	Morgan Creek Downstream	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	1	0
MT13	12	Morgan Creek Downstream	KLEBSIELLA	1	*Sphingobacterium multivorum*	1	0	1	0
MT14	13	Morgan Creek Downstream	KLEBSIELLA	1	*Sphingobacterium multivorum*	1	0	1	0
NT01	12	Jordan Lake	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	1	0
NT02	12	Jordan Lake	KLEBSIELLA	1	*Sphingobacterium multivorum*	1	0	1	0
NT03	12	Jordan Lake	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	1	0
NT04	12	Jordan Lake	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	0	1
NT05	12	Jordan Lake	KLEBSIELLA	1	*Sphingobacterium multivorum*	1	0	1	0
NT06	13	Jordan Lake	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	0	1
NT07	13	Jordan Lake	KLEBSIELLA	1	*Ralstonia insidosa*	1	0	0	1
NT08	13	Jordan Lake	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	1	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
OE02	8	H1 GI + MICU	ECOLI	0	NO MALDI	1	0	1	0
OE03	8	H1 GI + MICU	ECOLI	0	NO MALDI	1	1	0	0
OE04	9	H1 GI + MICU	ECOLI	0	NO MALDI	1	1	0	0
OE05	9	H1 GI + MICU	ECOLI	0	NO MALDI	1	0	0	1
OE06	10	H1 GI + MICU	ECOLI	0	NO MALDI	1	1	0	0
OE07	10	H1 GI + MICU	ECOLI	0	NO MALDI	1	1	0	0
OE08	10	H1 GI + MICU	ECOLI	0	NO MALDI	1	0	1	0
OE09	10	H1 GI + MICU	ECOLI	0	NO MALDI	1	1	0	0
OE11	13	H1 GI + MICU	ECOLI	0	NO MALDI	1	1	0	0
OT01	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT02	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT02 - TURQ	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT03	8	H1 GI + MICU	KLEBSIELLA	1	*Ochrobactrum anthropi*	1	0	1	0
OT04	8	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	1	1	0	0
OT05	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT06	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella oxytoca*	1	1	0	0
OT07	8	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT08	9	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii / werkmanii*	1	1	0	0
OT09	9	H1 GI + MICU	KLEBSIELLA	1	*Serratia marcescens*	1	1	0	0
OT10	9	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT11	9	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT12	9	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT13	10	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	1	1	0	0
OT14	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT14 - Turq	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT15	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT16	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT16 - TURQ	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
OT17	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT17 - TURQ	10	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT18	11	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT19	12	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
OT20	12	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	1	1	0	0
OT21	12	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	1	1	0	0
OT22	12	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	1	1	0	0
OT23	12	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	1	0	0
OT23 - PINK	12	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	1	0	0
OT24	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT24 - TURQ	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT25	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT25 - TURQ	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT26	12	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT27	12	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
OT28	13	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	0	0	1
OT28 - PINK	13	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
OT29	13	H1 GI + MICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	0	1	0
OT30	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT31	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT31 - TURQ	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT32	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT32 - TURQ	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT33	13	H1 GI + MICU	KLEBSIELLA	1	*Citrobacter freundii*	1	1	0	0
OT34	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
OT34 - turq	13	H1 GI + MICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
PE01	8	H2 Burn + Lab + ICU	ECOLI	1	*Escherichia coli*	1	0	1	0
PE02	9	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	1	0	0
PE03	9	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE04	9	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE05	9	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE06	9	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE07	9	H2 Burn + Lab + ICU	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
PE08	10	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	0	1
PE09	10	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE10	10	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE11	10	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE12	10	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE13	11	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	0	1
PE14	11	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	0	1
PE15	11	H2 Burn + Lab + ICU	ECOLI	1	*Klebsiella pneumoniae*	1	0	1	0
PE16	11	H2 Burn + Lab + ICU	ECOLI	0	NO MALDI	1	0	1	0
PE17	12	H2 Burn + Lab + ICU	ECOLI	1	*Klebsiella pneumoniae*	1	0	1	0
PT01	8	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
PT02	9	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
PT03	9	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
PT03A	9	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
PT06	10	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella oxytoca*	1	0	0	1

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
PT07	10	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
PT07A /NF	10	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	1	0	0
PT08	10	H2 Burn + Lab + ICU	KLEBSIELLA	0	NO MALDI	1	0	0	1
PT09	10	H2 Burn + Lab + ICU	KLEBSIELLA	0	NO MALDI	1	0	1	0
PT10	10	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	0	1
PT11	11	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT11/ NF	11	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT12	11	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT13	11	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT14	11	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT15	11	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT16	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
PT17	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	0	1	0
PT18	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Klebsiella pneumoniae*	1	1	0	0
PT19	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	0	0	1
PT20	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Cronobacter turicensis*	1	0	0	1
PT21	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	0	0	1
PT22	12	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Citrobacter amalonaticus / farmeri*	1	0	0	1
PT23	13	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
PT24	13	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	0	0
PT25	13	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	0	0	0	0
PT26	13	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
PT27	13	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
PT28	13	H2 Burn + Lab + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	0	0	0	0
QE01	8	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE02	8	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE03	8	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE04	9	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	0	1
QE06	10	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	0	1
QE08	10	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	0	1
QE10	10	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE12	10	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE14	12	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE15	12	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QE17	12	H3 Heart + ICU	ECOLI	0	NO MALDI	1	0	1	0
QT01	9	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
QT02	9	H3 Heart + ICU	KLEBSIELLA	0	NO MALDI	1	1	0	0
QT02A	9	H3 Heart + ICU	KLEBSIELLA	0	NO MALDI	1	1	0	0
QT03	9	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
QT04	9	H3 Heart + ICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	0	1	0
QT05	9	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	1	0	0
QT06	9	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	1	0	0
QT07	9	H3 Heart + ICU	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
QT08	9	H3 Heart + ICU	KLEBSIELLA	1	*Staphylococcus epidermidis*	1	0	1	0
QT09	9	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
QT10	10	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	0	1
QT11	10	H3 Heart + ICU	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
QT13	12	H3 Heart + ICU	KLEBSIELLA	0	NO MALDI	1	0	1	0
QT14	12	H3 Heart + ICU	KLEBSIELLA	1	*Stenotrophomonas maltophilia*	1	0	1	0
QT15	12	H3 Heart + ICU	KLEBSIELLA	1	*Pseudomonas putida*	1	0	1	0

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
QT16	12	H3 Heart + ICU	KLEBSIELLA	0	NO MALDI	1	0	1	0
RE02	8	H4 ER + Neuro	ECOLI	1	*Aeromonas hydrophila / caviae*	1	1	0	0
RE03	9	H4 ER + Neuro	ECOLI	1	*Serratia marcescens*	1	0	0	1
RE04	9	H4 ER + Neuro	ECOLI	1	*Ralstonia mannitolityca*	1	1	0	0
RE05	9	H4 ER + Neuro	ECOLI	1	*Citrobacter braakii / werkmanii*	1	1	0	0
RE06	9	H4 ER + Neuro	ECOLI	0	NO MALDI	1	0	1	0
RE07	9	H4 ER + Neuro	ECOLI	1	*Citrobacter amalonaticus*	1	0	1	0
RE08	10	H4 ER + Neuro	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
RE09	10	H4 ER + Neuro	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
RE11	10	H4 ER + Neuro	ECOLI	1	*Ochrobactrum anthropi*	1	0	1	0
RE13	12	H4 ER + Neuro	ECOLI	1	*Enterobacter cloacae / asburiae*	1	1	0	0
RE14	12	H4 ER + Neuro	ECOLI	0	NO MALDI	1	0	1	0
RE15	13	H4 ER + Neuro	ECOLI	1	*Citrobacter freundii*	1	1	0	0
RE16	13	H4 ER + Neuro	ECOLI	1	*Stenotrophomonas maltophilia*	1	0	1	0
RE17	13	H4 ER + Neuro	ECOLI	1	*Citrobacter werkmanii*	1	0	0	1
RE18	13	H4 ER + Neuro	ECOLI	0	NO MALDI	1	0	1	0
RT01	8	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	0	1	0
RT03	8	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	1	0	0
RT04	8	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	0	0	1
RT05	9	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	1	0	0
RT06	9	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	1	0	0
RT07	9	H4 ER + Neuro	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	1	0	0	1
RT08	9	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	1	0	0
RT09	9	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
RT10	9	H4 ER + Neuro	KLEBSIELLA	1	*Raoultella planticola / ornithinolytica*	1	0	0	1
RT11	10	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	0	1	0
RT12	10	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	1	0	0
RT13	10	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	0	0	1

KPC Code	Week	Site	KPC ID	MALDI – TOF	MALDI-TOF Result	Modified Hodge Test	KPC (+) Producer	KPC(-R) Intermediate	KPC (-) Negative
RT14	10	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	0	0	1
RT15	10	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	0	1	0
RT17	12	H4 ER + Neuro	KLEBSIELLA	1	*Serratia marcescens / odifera*	1	0	1	0
RT18	12	H4 ER + Neuro	KLEBSIELLA	1	*Citrobacter amalonaticus*	1	0	0	1
RT19	12	H4 ER + Neuro	KLEBSIELLA	1	*Serratia marcescens*	1	0	1	0
RT20	12	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	0	1	0
RT21	12	H4 ER + Neuro	KLEBSIELLA	1	*Klebsiella oxytoca*	1	1	0	0
RT22	13	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
RT23	13	H4 ER + Neuro	KLEBSIELLA	0	NO MALDI	1	1	0	0
RT24	13	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
RT25	13	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0
RT26	13	H4 ER + Neuro	KLEBSIELLA	1	*Enterobacter cloacae / asburiae*	1	1	0	0

APPENDIX 5 – KPC MALDI-TOF MS AND VITEK 2 RESULTS

S = Susceptible I = Intermediate R = Resistant based on MIC R = Resistant based on other phenotypic properties (VITEK Software Determined)*

KPC(+) = Positive KPC Production KPC(R-) = "Intermediate" KPC production KPC(-) = Negative for KPC Production (.) = Not applicable

KPC Code	Site	MALDI-TOF Result	Cefpodoxime		Imipenem		Hodge Results
			MIC	S/I/R	MIC	S/I/R	
KT16	Secondary Effluent	*Escherichia coli*	<=0.25	S	2*	S	KPC(-)
KT24	Secondary Effluent	*Klebsiella pneumoniae*	2	R*	4	R*	KPC(-R)
KT35A	Secondary Effluent	*Enterobacter cloacae / asburiae*	>=8	R	8	I	KPC(-R)
KT35B	Secondary Effluent	*Enterobacter cloacae / asburiae*	>=8	R	8	I	KPC(-R)
LT02	Morgan Creek Upstream	*Ochrobactrum anthropi*	>=8	R	4	S	KPC(-R)
LT04	Morgan Creek Upstream	*Ochrobactrum anthropi*	>=8	R	4	S	KPC(-R)
LT05	Morgan Creek Upstream	*Ochrobactrum anthropi*	4	I	2	S	KPC(-R)
LT07	Morgan Creek Upstream	*Ochrobactrum anthropi*	>=8	R	4	S	KPC(-R)
LT10	Morgan Creek Upstream	*Sphingobacterium multivorum*	>=8	R	>=16	R	KPC(-)
LT11	Morgan Creek Upstream	*Sphingobacterium multivorum*	>=8	R	>=16	R	KPC(-R)
MT04	Morgan Creek Downstream	*Pseudomonas chororaphis / fluorescens*	>=8	R	>=16	R	KPC(-R)
MT05	Morgan Creek Downstream	*Ochrobactrum anthropi*	>=8	R	2	S	KPC(-)
MT06	Morgan Creek Downstream	*Ochrobactrum anthropi*	>=8	R	2	S	KPC(-R)
MT07	Morgan Creek Downstream	*Ochrobactrum anthropi*	>=8	R	2	S	KPC(-)
MT08	Morgan Creek Downstream	*Stenotrophomonas maltophilia*	Not of clinical significance				KPC(-R)
MT09	Morgan Creek Downstream	*Ochrobactrum anthropi*	>=8	R	2	S	KPC(-)
MT11	Morgan Creek Downstream	*Elizabethkingia meningoseptica*	>=8	R	>=16	R	KPC(-R)
MT12	Morgan Creek Downstream	*Stenotrophomonas maltophilia*	Not of clinical significance				KPC(-R)
MT13	Morgan Creek Downstream	*Sphingobacterium multivorum*	4	I	>=16	R	KPC(-R)
NT01	Jordan Lake	*Stenotrophomonas maltophilia*	Not of clinical significance				KPC(-R)
NT02	Jordan Lake	*Sphingobacterium multivorum*	>=8	R	>=16	R	KPC(-R)
NT03	Jordan Lake	*Stenotrophomonas maltophilia*	Not of clinical significance				KPC(-R)
NT05	Jordan Lake	*Sphingobacterium multivorum*	>=8	R	>=16	R	KPC(-R)
NT06	Jordan Lake	*Ochrobactrum anthropi*	1	S	<=1	S	KPC(-)
NT07	Jordan Lake	*Ralstonia insidiosa*	4	I	4	S	KPC(-)

KPC Code	Site	MALDI-TOF Result	Cefpodoxime		Imipenem		Hodge Results
			MIC	S/I/R	MIC	S/I/R	
NT08	Jordan Lake	*Stenotrophomonas maltophilia*	Not of clinical significance				KPC(-R)
RT04	H4 ER + Neuro	*Enterobacter cloacae/asburiae*	4	I	4	S	KPC(-)
RT07	H4 ER + Neuro	*Raoultella planticola/ornithinolytica*	>=8	R	8	I	KPC(-)
RT10	H4 ER + Neuro	*Raoultella planticola/ornithinolytica*	>=8	R	4	S	KPC(-)
RT18	H4 ER + Neuro	*Citrobacter amalonaticus*	>=8	R	4	S	KPC(-)

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